Development of a synthetic sensor system for the detection of infectious and inflammatory signals

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Abstract

Bacterial infections are a major challenge, e.g. upon implant transplantation. Especially the formation of biofilms protects the bacteria within a polysaccharide matrix from effective treatment with antibiotics. In order to prevent biofilm formation anti-infective treatments have to be performed early with onset of infection. Inspired by synthetic biological devices this study aimed at engineering a cellular device which can detect infection or inflammation derived signals and transmit these signals to synthetic cassettes. Here, activation of the type I interferon (IFN) system was chosen as a physiological hallmark for viral and bacterial infections as well as a marker for inflammation. To rewire infection related input signals to synthetic cassettes the synthetic doxycycline dependent transactivator gene (tTA, Tet-off) was integrated into the endogenous murine Mx2 chromosomal locus. This locus has been assigned to be specifically induced by type I IFN. Integration of a single copy was performed using CRISPR/Cas9 assisted homologous recombination in murine embryonic stem (mES) cells. Subsequently the IFN signalling was coupled to luciferase expression by integration of a transactivator dependent expression cassette. The engineered ES cells displayed an attenuated IFN response as a consequence of endogenous regulatory mechanisms governing in stem cells. Upon differentiation these cells displayed high induction levels of reporter expression which could be reduced to basal levels by addition of doxycycline even with ongoing IFN signalling being present. Introduction of an autoregulatory circuit could increase the sensitivity without losing the ability of downregulation reporter expression in presence of doxycycline. In an alternative approach the tTA transactivator was integrated into a bacterial artificial chromosome (BAC) vector compromising about 150kb of the cellular Mx2 locus. Stable integration of the modified BAC vector into NIH/3T3 cells displayed a higher sensitivity and an overall higher luciferase expression compared to the single copy as obtained with the CRISPR/Cas9 approach. These systems could detect pathophysiological IFN concentrations *in vivo* and still preserved their ability to be regulated in presence of doxycycline. Finally, rewiring of infection signals to expression of Il10, an anti-inflammatory cytokine, was achieved. Together, this study represents the proof of concept for the functional rewiring of infectious signals to reporter and anti-inflammatory cues and offers great potential with regard to translational approaches.

Zusammenfassung

Bakterielle Infektionen sind nach wie vor eine große medizinische Herausforderung insbesondere bei der Verwendung von Implantaten. Ein Problem ist die Formation von Biofilmen. Diese Schutzschicht schützt die Bakterien vor dem Wirkungsmechanismus von Antibiotika. Um Biofilmformation zu verhindern, müssen Behandlungen zu einem frühen Zeitpunkt einsetzen. Der Fokus dieser Studie lag auf der Etablierung eines zellulären Systems, das infektiöse und inflammatorische Signale wahrnimmt und auf synthetische Expressionskassetten weiterleitet. Hier wurde das Typ I Interferon genutzt, das sowohl bei viralen und bakteriellen Infektionen als auch bei inflammatorischen Prozessen induziert wird. Um den durch Interferon aktivierten Signalweg zu synthetischen genetischen Kassetten umzuleiten, wurde das Gen für den Doxycyclin abhängigen synthetischen Transaktivator tTA (Tet-off) mittels homologer Rekombination und CRISPR/Cas9 in den endogenen murinen chromosomalen Mx2 Lokus in embryonalen Stammzellen integriert. In einem weiteren Schritt wurde die Interferon vermittelte Expression des Transaktivators an einen Luciferasereporter gekoppelt. Im pluripotenten Zustand zeigten die embryonalen Stammzellen eine geringe Luciferaseexpression in Anwesenheit von Interferon, die vermutlich auf endogene regulatorische Mechanismen in Stammzellen zurückzuführen ist. Im Zuge der Differenzierung der Zellen und nach deren Immortalisierung konnte die Reporterexpression jedoch deutlich gesteigert werden. Die synthetischen Kassetten erwiesen sich als funktional und konnten durch Zugabe von Doxycyclin vollständig abgeschaltet werden, selbst in Anwesenheit von Interferon. Die Kopplung mit einem autoregulatorischen Regelkreis zeigte eine Erhöhung der Reporterexpression durch die Amplifikation des Transaktivators, ohne den Doxycyclin abhängigen Regulationsmechanismus zu beeinflussen. In einer anderen Vorgehensweise wurde der Transaktivator in einen ca. 150kb des endogenen Mx2 Lokus umfassenden ‚Bacterial Arificial Chromosome' (BAC)-Vektors integriert und stabil in murine NIH/3T3 Fibroblasten eingebracht. Diese Zellen zeigten eine deutlich erhöhte Luciferaseexpression in Anwesenheit von Interferon, vermutlich aufgrund erhöhter Kopienzahlen und damit eine erhöhte Sensitivität. Beide Systeme waren nach Transplantation in die Maus in der Lage pathophysiologische Konzentrationen von Interferon zu detektieren; auch *in vivo* konnte das Signal mittels Doxycyclin abgeschaltet werden. Schließlich wurde das Signal zur Induktion eines anti-inflammatorischen Zytokins (Il10) eingesetzt. Das funktionelle Umleiten von

Infektions-induzierten Signalen auf synthetische Kassetten konnte mittels dieser beiden Systeme erfolgreich umgesetzt werden und eröffnet damit die Möglichkeit neuer therapeutischer Ansätze.

1.Introduction

1.1 Chronic infections and implants

Infection is defined as the invasion of a host´s organism by disease-causing organisms, their multiplication, and the reaction of the host tissues to the organism and the toxins they produce (Signore, 2013). The organisms which lead to an infection include viruses, prions, bacteria, viroids and larger organisms like parasites and fungi. In mammalian, host´s infection lead to an innate immune response, often involving inflammation, followed by an adaptive response. Therefore infection is usually always associated with inflammation (Signore and Glaudemanns, 2011).

Acute bacterial infections are assumed to involve planktonic bacteria, existing as a single, independent cell (Bjarnsholt, 2013). These form of bacterial infection is generally treatable with antibiotics. Chronic bacterial infections involve the formation of a so called biofilm. Biofilms are a structured community of bacterial cells enclosed in a self-produced polymeric matrix adherent to a surface (Costerton et al., 1999). Within these biofilms bacteria are joined together and produce an extracelluar matrix that contains many different types of polymeric substances, including proteins, DNA and polysaccharides. Bacteria in the biofilm withstand high doses of antibiotics that would kill the planktonic cells (Ciofu et al., 2017). Additionally their tolerance of host defences is also dramatically increased (Roilides et al., 2015). As these biofilms often form on surfaces theses chronic infections are often found on implants.

Apart from cosmetic applications many medical implants serve as prosthetics to restore missing body parts and thereby restore their function e.g. hip implants, dental implants. Others are used to deliver medications or monitor body functions. The material used for fabrication is quite variable. Some are made from skin or bone while others are derived from plastic, metal or ceramic.

Along with the growing number of implant placement there is also a growing number of reports of associated infections occurring (Sanz and Chapple, 2012). These infections mostly arise from biofilm formation on the surface of the implant which are highly susceptible to bacterial infections due to the compromised host defense (Zimmerli et al., 1982).

To avoid attachment of bacteria on the implant surface strategies have been developed to combat initial adhesion. The surface chemistry of implants can change the efficiency of

bacterial adhesion and proliferation capacity. These factors include charge, hydrophobicity, surface roughness or physical configuration (Katsikogianni and Missirlis, 2004). However, these properties should preserve the biocompatibility of the implant itself.

Modifications on the surface of the implant material have also been considered as a strategy to combat bacterial adhesion (Nagano-Takebe et al., 2014). Additionally antibiotic release devices have been established to combat bacterial growth on implants (Gimeno et al., 2015). However these systems are incipient stages and have several disadvantages. The amount of compound delivered by these fixed systems is limited. There would be no chance of refreshing due to the inaccessibility of the implant. Additionally, the concentrations which are delivered by these systems at a given time might not be sufficient to cope with the bacterial infection. With respect to antibiotic usage an inappropriate dosage might promote resistance of microorganisms which would reduce treatment options in the end. Furthermore, most of these systems rely on the uncontrolled release of the compound. This leads to a very high local concentration in the beginning followed by a rapid clearance of the drug. Although the release might be continuous over a period of time it might be not needed at this particular moment.

Although these recent developments could improve the implant materials to a certain extent, the so far established systems to interfere with implant associated infections still display particular disadvantages on their own.

1.2 Synthetic biology

Synthetic biology is a rising inter-disciplinarily field combining applications of cellular biology and engineering at the same time. The main aim of synthetic biology is to re-design and re-engineer biological systems (e.g. cells) which is usually achieved by modification of the cells' genetic information (König et al., 2013). Biotechnological principles take advantage of the genetic information with regard to economic use. Specific genetic information is transferred from one organism to another and provides the recipient with new useful features. The advances in molecular, cell and systems biology offer the possibility to design and construct new biological parts, devices and systems on a modular basis. Here, synthetic biology focuses on the construction of so called core components that can be modulated and assembled to more complex units (Church et al., 2014). Key elements in synthetic biology are genetic

switches. Transgene expression can be synthetically modulated by the use of external signals like the addition of a metabolic compound, a small molecule or by physical signals like light of a specific wavelength or electromagnetic fields (Kemmer et al., 2010; Gitzinger et al., 2012; Kennedy et al., 2010)

Gene expression can be regulated by specific protein-protein or protein-DNA interaction to form transcription activator complexes. This simple principle is shared within all organisms which at the same time displays a huge diversity of different stimulus responsive protein-protein or protein-DNA interactions. Synthetic biology takes advantage of this diversity in order to functionally reconstitute synthetic transcriptional activator complexes. By fusing transcriptional activator or silencer domains to an inducible DNA-binding protein synthetic transcriptional activator have been designed which can be used to conditionally induce (trans)gene expression from the cognate promoter (Wang et al., 2012; Weber et al., 2008; Rinaudo et al. 2007). A variety of these genetic switches have been established in mammalian cells to get control on transgene expression. These include switches responsive to drugs (Fussenegger et al., 2000), metabolites (Hartenbach et al., 2007), vitamins (Weber et al., 2007) or even light (Yazawa et al., 2009).

The variety of responsive elements to specific signals offers the possibility to implement these into existing cellular pathways. By this, the genetic switches have been connected to endogenous signals so that they can act in an autonomous and seamless fashion. As a general principle example the therapeutic gout device can be mentioned which detects pathophysiological concentrations of urate in the bloodstream and declines these automatically to physiological levels (Kemmer et al., 2010).

Different examples using a similar setting have shown that the connection of synthetic circuits to endogenous pathways is a powerful tool to interfere with biological processes. The bandwidth of applications does not seem to be limited in this regard. Devices have been established to sense high glucose level in a model of insulin resistance (Ye et al., 2017) or synthetic circuits have been evolved for the artificial insemination (Kemmer et al., 2011) of cows just to mention some of them.

The modularity principles of synthetic biology allow the functional interconnection of different building blocks leading to establishment of programmable logic gates (Kramer et al., 2004). These gates consist of several transcriptional units which are interconnected in a circuit encoding principle. By this the genetic circuit can perform AND-gate logic signal processing

according to the availability of the cognate input signal (Ausländer and Fussenegger, 2013). This chaining cascade of building blocks enhances the specificity of a synthetic circuit to combinations of endogenous signals. Furthermore, this renders the circuit more predictable in terms of output signals – a common aim of synthetic biology. Best exemplified is such a circuit in the work of (Schukur et al.2015).

There is an accelerated progression in the field of synthetic biology. Within several years this field of biology has engineered cellular systems which can respond to a variety of diverse signals dependent on their synthetic circuit. Examples have been given that cells can be controlled by different wavelengths, magnet fields or even with smart phone devices nowadays (Shao et al., 2017). Synthetic cellular circuits for therapeutic approaches display great potential for translational medicine. Cells can be engineered with the desired patients´ need and be specially geared to specific disease parameters. So far, these systems are proof of concept studies and have not been applied for clinical trials. This might change within years with progress in terms of biosafety of cell therapy.

1.3 Tet-Systems

In 1992 Gossen and Bujard described the tetracycline-controlled transcriptional regulation system commonly known as Tet-off system for the first time (Gossen and Bujard, 1992). Before this system emerged, attempts to control gene expression by eukaryotic promoters had failed due to varieties of side effects (Lee et al., 1988). Others like lacR/O-based system in which gene expression is dependent on presence of isopropyl β-D-thiogalactopyranoside (IPTG) are ineffective due to slow and moderate induction rates of transgene expression.

The tetracycline regulatory system is derived from the Tn*10*-specific tetracycline-resistance operon of *E. coli*. Here, the tetracycline repressor (*tet*R) directly represses the transcription of genes mediating tetracycline resistance. In presence of tetracycline TetR binding to its cognate DNA sequences is impeded. Thereby transcription of the resistance genes is mediated. The TetR repressor was fused to the C-terminal domain of VP16 from herpes simplex virus (HSV). VP16 is a transcriptional regulatory protein known to induce the expression of immediate early genes in HSV. The fusion of these two different domains constitutes the synthetic transcription factor tTA. Target gene expression by tTA is mediated from tetracycline-response promoter elements (TREs). These elements contain repetitive *tet*O sequences which

are the cognate DNA binding sequences for TetR and mostly a minimal CMV promoter devoid of transcription factor binding sequences.

In the absence of tetracycline the tTA transactivator binds to TRE and activates target gene expression from the minimal CVM promoter. Addition of tetracycline impedes tTA binding and thereby represses gene expression (Figure 1A).

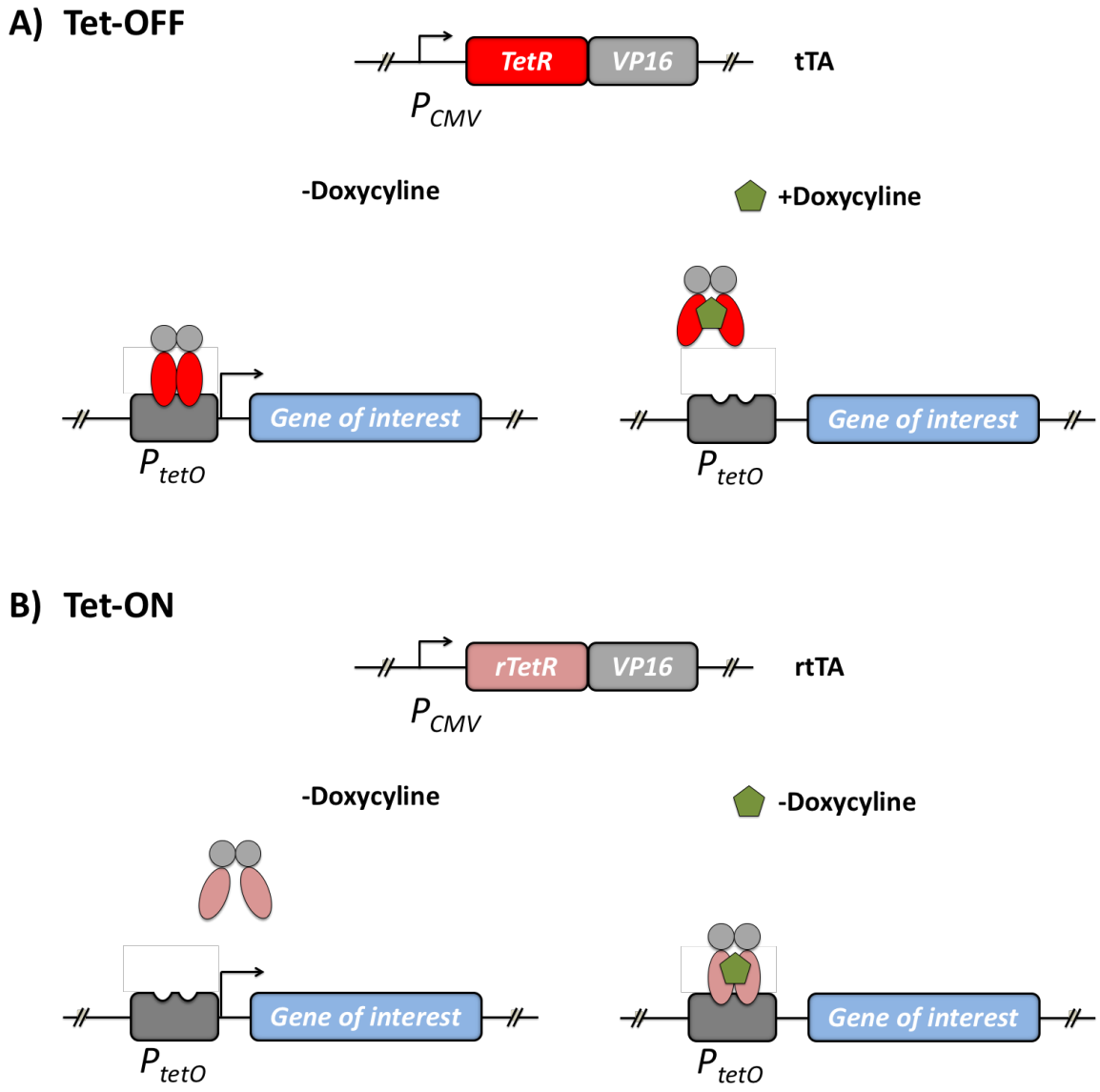

Figure 1: Schematic representation of doxycycline controlled transcriptional regulatory systems.

A) In the Tet-OFF system the constitute expression of the tTA transactivator from a CMV promoter drives expression in absence of doxycycline. The tTA transactivator can bind to its cognate DNA binding sequences located within PtetO. Expression of the gene of interest is induced via the VP16 domain of the tTA transactivator. In presence of doxycycline the tTA transactivator cannot bind to TetO sequences to drive target gene expression. B) In the Tet-ON system the constitutive expressed reverse transactivator rtTA cannot bind to TetO DNA sequences in absence of doxycycline. The gene of interest is not expressed. In presence of doxycycline conformational changes are induced and the rtTA can bind to its cognate DNA sequences. This results in target gene expression.

The Tet system itself has been extensively optimized over the years. In 1995 the Tet-On system was established by using a mutant TetR version designated as reverse transactivator (rtTA) (Gossen et al., 1995). This version of transactivator binds to TREs in the presence of tetracycline and thereby inducing target gene expression (Figure 1B).

Different mutants of rtTA have been developed showing improved functionality with regard to stability, background expression, tetracycline dependent response or toxic effects of the transactivator (Baron et al., 1997; Urlinger et al., 2000). Additionally TREs were optimized in order to reduce background expression and increase target gene expression (Agha-Mohammadi et al., 2004; Loew et al., 2010).

The tetracycline expression system by Gossen and Bujard is dependent on a constitutive expression of the tTA transactivator from a functional human cytomegalovirus (CMV) promoter. Here, tetracycline prevents the expression of the gene of interest by impeding the DNA binding ability of the tTA transactivator to its cognate DNA *tetO* seuqences. In this system the expression of the gene of interest is dependent on the expression of the tTA expression from the CMV promoter. To increase transgene expression so called autoregulatory systems were established (Schockett et al., 1995). In these autoregulatory systems, transactivator expression is controlled from *TetO* promoters. Here, the expression of the transactivator is regulated by itself and in turn leads to an autoregulated amplification. In turn, expression of reporter expression which is dependent on transactivator binding is also increased (Heinz et al., 2013). These self amplifying systems have been described fot the tTA (Unsinger et al., 2001) as well as for the rtTA transactivator (Schucht et al., 2010).

The Tet-system offers the possibility for conditional manipulation of gene expression and has been used in many different approaches for *in vitro* and *in vivo* studies. (Vincent et al., 2006; Vieyra and Goodell, 2007; Avery, 2011; Heindorf and Hasan, 2015)

1.3 Type I IFNs

The ability of cells to respond to either infectious or inflammatory input signals is a requirement for cellular survival and given to almost all cell types. The presence of e.g pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) is recognised by cells of the innate immune system. Although these molecules are recognised by different receptors the subsequent cellular response mediates a similar anti-

infective or anti-inflammatory state in the end. Among the secretion of anti-inflammatory cytokines also IFNs belong to the molecules mediating these subsequent effects. In the following chapters the role of IFN with regard to infections will be highlighted.

1.3.1 An overview

Almost 60 years ago type I IFNs was first described mediating viral interference (Isaacs and Lindenmann, 1987). Type I IFNs can be classified into different subgroups: IFN-α encoded by 13 to 14 subfamily genes, IFN-β encoded by a single gene and others like IFN-ω, IFN- ε, and IFN-κ (Hardy et al., 2004). Among these IFN-α and IFN-β genes are the best characterized and most broadly expressed and will be emphasized in the following chapters.
Type I IFNs represent the first line of defence against invading pathogens (Pietras et al., 2006). Their expression is induced by different cellular receptors which leads to their subsequent secretion. After binding to their cognate receptor which is composed of two chains, IFNAR1 and IFNAR2, they activate intracellular antimicrobial programmes and influence the development of innate and adaptive immune response (Tough, 2004). This is mediated by the expression of so called interferon stimulated genes (ISGs). These genes confer an anti-infectious state thereby limiting the pathogen spread. In the next chapters this will be explained in more detail.
Although type I IFNs have been described in viral interference first, they also mediate cellular response against bacteria, parasites, fungi either direct or indirectly through the induction of other mediators (Pietras et al., 2006; Lauvau et al., 2015). By this, type I IFNs can be assigned as a general marker for an infectious state.

1.3.2 Regulation of type I IFN expression

The expression of type I IFNs is mostly induced upon recognition of pathogen-associated molecular pattern (PAMPs). These are evolutionary conserved structures of pathogens derived from different origin (Silhavy et al., 2010). They include e.g. bacterial carbohydrates (lipopolysaccharide, mannose), nucleid acids (bacterial or viral DNA, RNA) or bacterial peptids (flagellin, microtubule). All these foreign structures are recognised by a large family of cellular pattern recognition receptors (PRRs). These include the membrane-bound toll-like receptors

(TLR) located at the cellular or endosomal membranes (Beutler et al., 2006) (Figure 2). Up to now 10 TLRs have been identified in humans every single one recognizing a distinct PAMP. While TLRs are membrane bound receptors they are not able to recognize intracellular pathogens and their derivatives. Here another set of PRRs guarantees pathogen recognition. These can be divided into retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) (Kanneganti et al., 2007; Loo and Gale, 2011). Both of these receptors transduce the signal in the presence of dsRNA whereas the length of the RNA dictates receptor specificity (Kato et al., 2008). Additionally these PRRs can recognize self-RNA derived from RNaseL nuclease activity thereby amplifying IFN signalling (Malathi et al., 2007). The third member for dsRNA recognition is IFN-inducible dsRNA–activated protein kinase (PKR) (Nallagatla et al., 2007). This kinase directly targets eukaryotic initiation factor elf2α inhibiting protein synthesis (Sadler and Williams, 2007).

Sensing of cytoplasmatic localized DNA is mediated by DNA-dependent activator of IFN-regulatory factors (DAI) which was the first cytosolic DNA sensor discovered (Takaoka et al., 2007). The asparatate-glutamate-any amino acid-asparatate/histidine (DExD/H) box-containing helicases emerged as important sensors in cytosolic DNA recognition. Here DDX41 should be highlighted because it interacts with the stimulator of interferon genes (STING) (Zhang et al., 2011) which is the main signalling adapter in the cellular DNA sensing pathway (Keating et al., 2011). All so far mentioned DNA sensor as well as RNA polymerase III, IFI16 and cytosolic GAMP synthase (cGAS) initiate the downstream signalling pathway via STING leading to the production of type I IFNs (Figure 2).

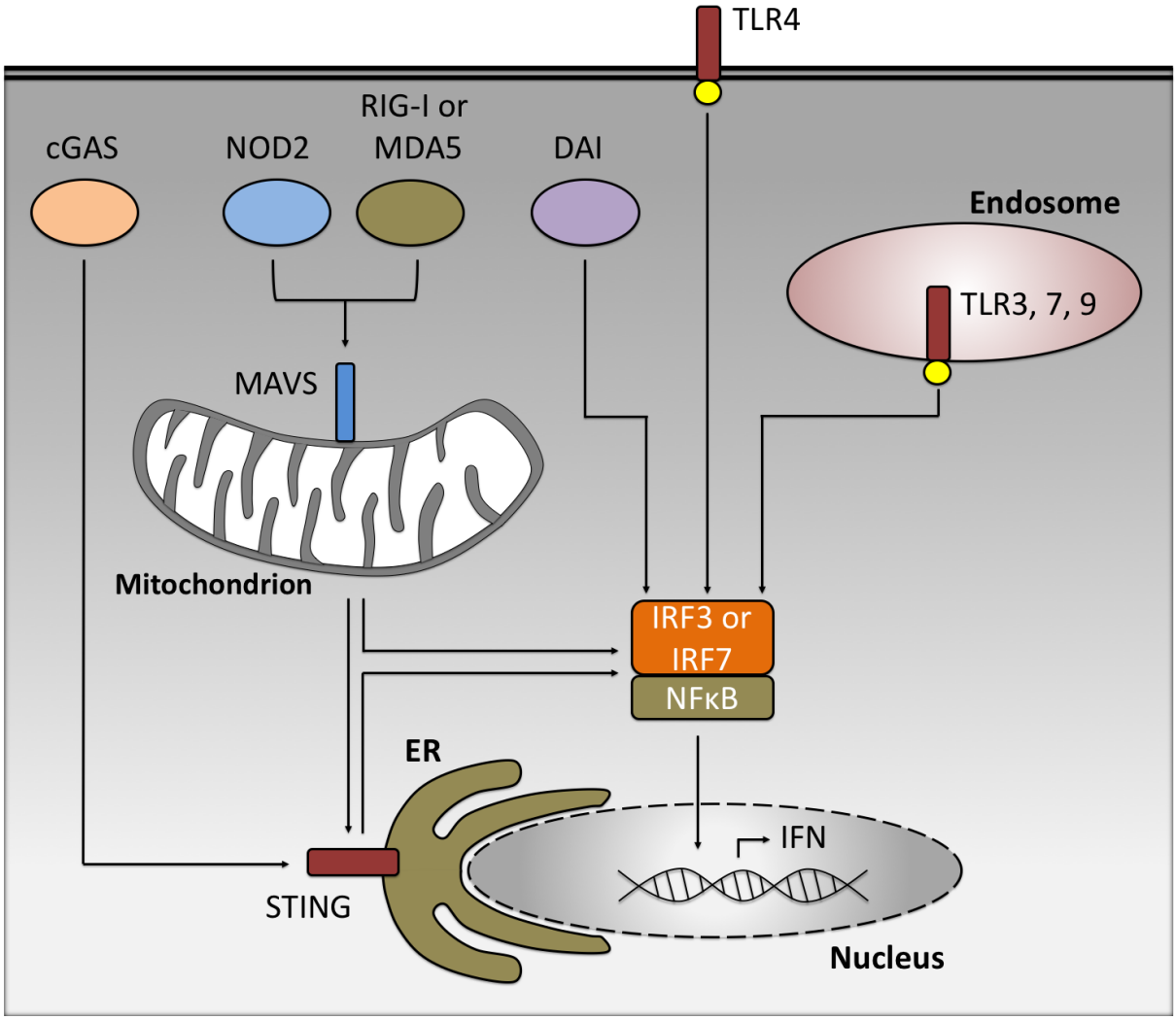

Figure 2: Schematic representation of pathways involved in type I IFN induction.

Recognition of microbial structures is mediated by a set of cell-surface and intracellular receptors. These include Toll-like receptors (TLR) and retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), DNA-dependent activator of interferon regulatory factors (IRFs) (DAI) or cytosolic GAMP synthase (cGAS). The presence and recognition of microbial pattern leads to the induction of type I IFN by several distinct signalling pathways. MAVS, mitochondrial antiviral-signalling protein; STING, Stimulator of interferon genes; NFκB, nuclear factor-κB; ER, endoplasmatic reticulum. Note that this is a representative scheme. For illustration purposes several molecules within signalling pathways where not shown.

Type I IFN expression is mainly regulated on the transcriptional level. After viral infection a coordinative binding of three sets of factors is taking place which recognize positive regulatory domains (PRDs) in IFN promoter regions: NF-κB, interferon regulatory factors (IRFs) and ATF-2/c-Jun form together with the architectural protein HMG the enhanceosome (Maniatis et al., 1998). This complex is essential for recruiting the transcriptional machinery and chromatin-remodelling proteins (Thanos and Maniatis, 1995; Struhl, 1998). Among the IRFs, IRF3 and IRF7 are the key regulators of type I IFN expression after viral infection.

IRF3 is constitutively expressed in a latent form in the cytosol whereas the amount of IRF7 is low but increases upon IFN stimulation (Lin et al., 1998; Marié et al., 1998) resulting in a positive feedback loop. An exception are plasmacytoid dendritic cells (pDCs) which are the main producers of interferon (Cella et al., 1999) due to their constitutive expression of IRF7 (Prakash et al., 2005). Both transcription factors translocate to the nucleus after phosphorylation by TANK-binding kinase 1 (TBK1) or IκB kinase (IKK) (Honda et al., 2006).
IRF7 can be assembled in homodimers or together with IRF3 in heterodimers. Each of these dimer complexes activates type I IFN genes. Whereas IRF3 is more potent in activating IFN-β rather than IFN-α, IRF7 activates both to the same extend (Sato et al., 1998). The fact that IRF7 has a short half-life (~0.5-1hrs) renders the activation process limited and transient (Sato et al., 2000).

1.3.3 Canonical type I IFN signalling cascades

Type I IFNs share the same heterodimeric transmembrane receptor called IFNα receptor (IFNAR). The receptor is composed of two subunits designated IFNAR1 and IFNAR2 (Uzé et al., 2007) and are constitutively expressed on most cell types (Noppert et al., 2007). Binding of type I IFNs to their cognate receptor transmits the signal to the nucleus to regulate gene expression mainly via the JAK/STAT pathway (Stark and Darnell, 2012). Both receptor subunits lack kinase activity and are therefore associated with their respective Janus kinase (JAK). IFNAR1 is pre-associated with tyrosine kinase 2 (Tyk2) and JAK1 contacts IFNAR2 (Prchal-Murphy et al., 2012). Ligand binding to IFNARs induces dimerization of the subunits which leads to a conformational change. Thereby IFNAR associated signal transducer and activator of transcription (STATs) are activated by phosphorylation (Su and David, 2000). STAT1 is phosphorylated by Jak1 whereas Tyk2 phosphorylated STAT2. This activation processes of these signalling molecules leads to their dissociation from the receptor subunits and dimerization. Together with IRF9 they form a complex called IFN-stimulated gene factor 3 (ISGF3) which translocates to the nucleus and binds to IFN-stimulated response elements (ISRE) located in promoter regions of ISGs (Platanias, 2005). Thereby they directly activate their expression (Figure 3).

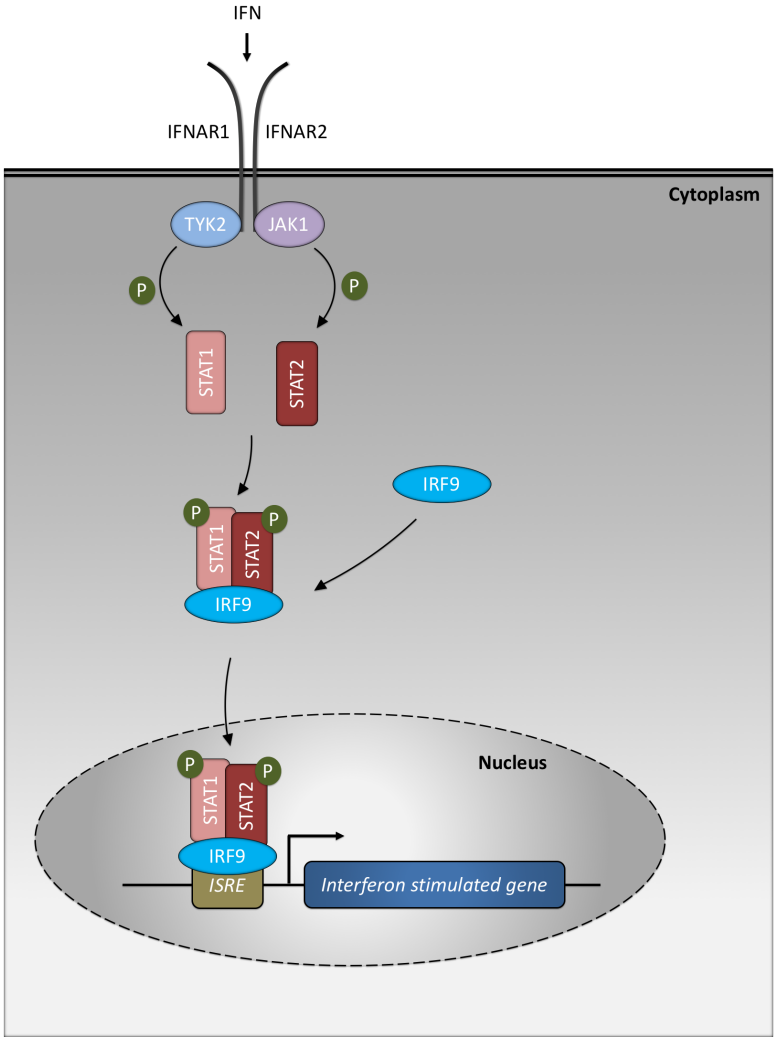

Figure 3: Schematic representation of canonical IFN signalling.

Binding of type I IFN to its cognate receptors (IFNAR1, IFNAR2) leads to dimerization of the two subunits and subsequent phosphorylation of receptor associated signal transducer and activator of transcription (STAT) by the janus kinase (JAK1) and tyrosine kinase (TYK2). Upon dimerization of phosphorylated STAT1 and STAT2, interferon regulatory factors 9 (IRF9) associated with the two molecules to form the IFN-stimulated gene factor 3 (ISGF3) complex. Translocation to the nucleus and binding to IFN-stimulated response elements (ISRE) induces the expression of IFN stimulated genes.

1.3.4 Ambivalent role of type I IFN mediated responses upon bacterial infection

Bacterial infections trigger IFN production mainly through the presence of bacterial nucleic acids or gram-negative cell wall compound LPS which are recognized by the innate immune receptors. IFN-β as well as IFNα4 belong to the first type I IFNs which are produced and trigger the expression of other type I IFN genes (Marié et al., 1998). The pathways which induce their production have been described earlier.

The role of type I IFN upon bacterial infection is quite ambivalent. Suppressive as well as stimulatory immunomodulatory processes have been reported to be mediated by type I IFN.

These might be context dependent but also dependent on the bacterial strain causing the infection.

The extracellular human pathogen *S. pyogenes* can cause invasive and deadly infections. It has been shown that mice deficient in IFNAR1 are more susceptible to *S. pyogenes* infection (Gratz et al., 2011). Here IFN mediates resistance against the bacterial strain by suppressing the transcription of IL-1β. The overexpression of this pro-inflammatory cytokine in an infection scenario causes hyper-inflammation and organ damage. The balancing of *IL1B* transcription counteracts these detrimental processes. Additionally, beneficial roles of IFNs have been assigned driving the production of several pro-inflammatory cytokines. Upon infection with the gram-negative bacterium *H. pylori* IFN stimulates a CXCL10 driven inflammation. The absence of this pro-inflammatory process causes increase *H.pylori* load in the stomach in a mouse model (Watanabe et al., 2010). Underlining these contributions of IFN to beneficial pro-inflammatory processes are reports suggesting that diminished production of pro-inflammatory cytokines, like TNF or IL-6, lead to an increased dissemination of *L. monocytogenes* in IFN signalling deficient mice (Kernbauer et al., 2013).

On the other hand a lot of data confirms the disadvantage of immunomodulatory effects of IFN upon bacterial infection. The intracellular pathogen and causative agent of tuberculosis, *M. tuberculosis*, establishes a systemic dissemination. Here the inhibition of IL-1 by IFN seems to be the key mechanism (Mayer-Barber et al., 2011). In contrast the progression of inflammatory processes driven by IFN in a lung infection with the gram-positive extracellular bacterium *S. aureus* causes enormous tissue damage. This is mediated by excessive leukocyte infiltration in lungs of infected mice (Martin et al., 2009).

These few examples should indicate that the immunomodulatory effects of IFNs can have both beneficial as well as detrimental effects. This may dependent on the bacterial strain itself causing the infection. However, there are also reports suggesting that the route of infection has a huge impact on the sobriety. Intraperitoneal or intravenous infections with *L. monocytogenes* suggested an unfavourable role of IFN whereas ingested pathogen-contaminated food had no impact at all (Pitts et al., 2016). This may also imply that IFN has different functions in different tissues or organs (Hedges et al., 2016).

While the processes mediated by IFN affect the pathogenicity dependent on the bacterial strain IFN, especially IFN-β, are the first cytokines produced upon bacterial infections.

1.5 Precise gene targeting via homologous recombination

Targeted transgene integration offers the possibility to study the expression in context of the cellular genome. By this so-called position effects can be controlled (Roberts et al., 2014). These are effects mediated by influence of the cellular DNA sequence and chromatin at the site of chromosomal integration (Bestor, 2000).

The process which mediates gene targeting is called homologous recombination. It is mainly used to repair harmful DNA lesions that occur after DNA double strand breaks.

The process of homologous recombination offers the possibility to precisely integrate transgenes in a sequence dependent fashion into the desired endogenous loci. This technique was initially used in the late 80´s to silence genes of interest in embryonic stem cells (Smithies et al., 1985; Mansour et al., 1988). This also gave rise to the accessibility of knock-out mice. This had revolutionized the analysis of gene function and was awarded by the Nobel Prize in Physiology and Medicine to their inventors in 2007.

The basic principle of gene targeting is based on homologous recombination between a targeting vector and the endogenous genomic locus of interest The targeting vector is usually composed to three different parts: a) a 5´ homology arm b) a positive selection cassette and c) 3´ homology arm. The efficiency of homologous recombination is mainly dependent on the length of the flanking homology arms but also on the cell-type itself. Longer homologous sequences increase the frequency of integration (Deng and Capecchi, 1992). Furthermore linearization of the target construct increases the frequency of recombination additionally (Thomas et al., 1986).

The technique was further expanded by the approaches using inducible or tissue restricted gene expression. For example the tetracycline transactivator (tTA) or reverse tetracycline transactivator (rtTA) was used to control calcineurin expression in neurons of the forebrain (Mansuy et al., 1998.). In this study, rtTA was placed downstream the CaMKIIα promoter and used to study memory. Furthermore CRE-recombinase/LoxP systems were integrated to particular tissues facilitating inducible gene expression in restricted sites (Tsien, 2016).

Gene targeting events occur at a low frequency of one event per 10^5 to 10^7 treated cells and relies on the endogenous homologous recombination pathway (Jasin and Berg, 1988). To increase gene targeting events at a desired locus DNA double strand breaks are usually introduced in close proximity to the target site. Several technologies have been used to direct

an endonuclease to a specific DNA locus and induce double strand breaks. These tools include Zinc Finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Gaj et al., 2013).

1.5.1 CRISPR/Cas9 system

The clustered, regulatory, interspaced, short palindromic repeats (CRISPR)/Cas9 system from *Streptococcus pyogenes* (also referred to as type II system initially described as a part of the bacterial innate immune system) has developed into a versatile tool for genome and epigenome editing (Jinek et al., 2012; Shen et al., 2013).

CRISPR elements are repetitive sequences of around 20 nucleotides in length found in specific genomic loci in the genome of bacteria and archaea. Originally these sequences are derived from invaded bacteriophages and serve as a part of the bacterial innate immune system to counteract infections comparable to a “genetic memory”. The immunization mechanism can be divided into three major steps, acquisition, biogenesis and interference (Carter and Wiedenheft, 2015). In the acquisition phase genetic material from the invading organisms is inserted into the CRISPR locus in form of so called spacer. Importantly the selection of DNA material is not a random process. It is dependent on a so-called protospacer adjacent motif (PAM) located in the genomic content of the invading organisms (Moijica et al. 2009). Spacers or protospacers are selected based on the presence of this PAM (Sorek et al, 2013). During biogenesis long transcripts encompassing the CRISPR array are produced and subsequently processed to form CRISPR-RNA (crRNA) (Charpentier et al., 2015). This crRNA guides the Cas9 endonuclease to its target sequence. The type II system additionally encodes a small RNA that is complementary to the crRNA known as trans-activating RNA (tracrRNA) which is required for Cas9 activity (Lim et al., 2016).

In the interference phase Cas9 recognizes PAM sequences within the genetic content of the cell. If complementary of the crRNA and the target sequence is given a conformational change in Cas9 positions its HNH and RuvC/RNaseH-like endonuclease domains to introduce DNA double strand breaks 3-4bp adjacent to the PAM within the crRNA and the complementary DNA sequence (Anders et al., 2014).

Whereas in the other two CRISPR systems multifunctional protein complexes contribute to DNA cleavage, the type II system relies on a single endonuclease Cas9. Additional re-

engineering by fusion the two RNA molecules into a “single-guide” RNA (sgRNA) made CRISPR/Cas9 a breakthrough technology of the 21th century (Jinek et al., 2012).
The simplicity of the system with regard to sequence specific targeting offers a huge potential for targeted integration of transgenes. Here CRISPR/Cas9 is “just” a tool to enforce endogenous processes like non-homologous end joining (NHEJ) or homologous recombination (HR) through endonuclease mediated DNA double strand breaks. Thereby CRISPR/Cas9 offers the possibility to target almost every sequence within a given genome.

1.6 Aim of this work

The introduction of synthetic expression cassettes into eukaryotic cells offers the possibility to control transgene expression using external triggers. These triggers could either be of physical or chemical nature.
In the present study, the aim was to engineer and validate a sensitive synthetic circuit that is able to specifically respond to physiological triggers that are released upon infection or inflammation. This should ensure transgene expression on demand without external adjustment. To achieve proof-of concept, such a circuit should be implemented and evaluated both, in stem cells and in differentiated cells. In particular, it should be tested if such engineered cells could be used to visualize infection, both in in vitro cell culture conditions as wells as upon transplantation of these modified cells in mice in vivo. With regard to the sensitivity of the synthetic circuit it should be attempted to amplify the output signal.
Finally, it should be tested if the established synthetic circuit could be used to rewire inflammatory input signals to an anti-inflammatory response. With the design and validation of a novel strategy towards controlled drug release this study aims at exploring a novel concept that might be of value to combat local infections such as implant associated infections.

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2. Materials and Methods

2.1 Equipment

Table 1: Equipment

Instruments	**Manufacturer**
Table top centrifuges	Eppendorf
	Heraeus Biofuge
Cooling centrifuge	Heraeus Biofuge fresco
	Sorval Superspeed RC5C
	Inflexible rotors: GSA, GS3, SS34
	Swinging rotor
Photometer	Nanodrop Spectrophotometer ND-1000
Gel electrophoresis systems	Gibco BRL horizontal gel electrophoresis apparatus
Micropipettes	Gibco
	Eppendort
Power supplies	Gibco BRL ST504
	Biorad Power PAC 200
Microscopes	Nikon TMS
	Leica Labovert FS
UV chamber	Hanau
Luminometer	Berthold Lumat LB 9501
Cell counter	Guava easy cite , Viacount
Cell culture incubators	Forma scientific water jacketed incubator
	Labotect C200
Safety benches	Sterile Gard Class II Type A/B3
	Herasafe, Heraeus HSP18
	Herasafe, Heraeus KS15
pH meter	Beckmann M340
Thermomixer	Eppendorf Thermomixer compact

Vortexer	Scientific industries Vortex genie 2
Deionized water supply	Milipore MiliQ
Electroporator	Biorad Gene Pulser and Pulse Controller
Thermocycler	T3 Thermocycler, Biometra
Precision Weighing	Sartorius
Microwave	Whirlpool
Waterbath	GFL
4°C refrigerator	Liebherr Comfort
-20°C freezer	Liebherr
-70°C freezer	Thermo Forma
In vivo biolumescence imaging machine	Xenogen IVIS system, Caliper IVIS2000
Gel documentation system	Intas
Flow cytometry	FACS calibur
RT-PCR system	LightCycler® 480 Real-Time PCR

2.2 Consumables

Table 2: Consumables

Material	**Supplier**
Bacterial petri dishes	Nunc
Cell culture plates (96well, 24well, 12well, 6well)	Nunc
Combi Tips (0.5ml, 2ml, 5ml)	Eppendorf
Kryogenic vials	Corning
Falcon tubes (15ml, 50ml)	Greiner bio-one
PCR tubes	Biozym
Pipette tips (10µl, 200µl, 1000µl)	Star Labs
Safe lock tubes (1.5ml, 2ml)	Eppendorf
Syringe filters (0.2µm and 0.45µm)	Sartorius
Tissue culture dishes	Corning, Greiner Bio-one
Luciferase measurement tubes (5ml)	Sarstedt

2.3 Chemicals and kits

Table 3: Chemicals and kits

Chemicals	**Supplier**	**Catalogue no.**
Decitabine	Sigma Aldrich	A3656-10MG
Doxycycline hyclate BioChemica	AppliChem GmbH	A29951,0025
IsoFlo	Albrecht GmbH	701-005-301
10mM dNTP Mix	Invitrogen	18427-013
2XRed PCR Master Mix	p.j.k.	302004
Lipofectamine 2000	Lifetechnologies	11668019
SYBR®Green PCR Master Mix	Lifetechnologies	4309155
mIL-10 ELISA	Biolegend	431411
RNase free DNase Set (50)	Qiagen	79254
NucleoBond® Xtra Maxi (100)	Macherey-Nagel	740.414.100
NucleoBond® BAC	Macherey-Nagel	740579
RevertAid First Strand cDNA Synthesis Kit 100rxn	Thermo Scientifc	K1622
RNeasy Mini Kit (250)	Qiagen	74106
MidoriGreen	Nippon Genetics	MG04
Expand Long Template PCR system	Roche	11681834001
IsoFlo®	Allbrecht GmbH	701-005-301
DNase I	Roche	11 284 932 001

2.4 Software and statistical analysis

This thesis was written using Microsoft Office 2011 specifically Microsoft Word and PowerPoint. qRT-PCR analysis was performed with LightCycler 480 SW1.5 and Microsoft Excel. *In vivo* bioluminescence data was analyzed with Living Image 2.60.1 software from Xenogen. Analysis of microscopic pictures was performed with ImageJ. Vector NTI AdvanceTM10 and SerialCloner stoftware was used for managing DNA and plasmid files. Chromas version 2.32 from Technelysium Pty Ltd and 4peaks was used to analyse DNA sequencing files. Literature

files was managed using Citavi5. GraphPad Prism Version 7.0b was used for data illustration and statistical analysis. Data between two experimental groups were analysed using Mann-Whitney test.

2.5. Sterilization

2.5.1 Sterilization by heat and filtration

Glass materials were sterilized by 180°C for 4 hours. All solutions were sterilized by filtration (0.22μm filter) or by autoclaving (20 minutes, 121°C, 1bar).

2.6 Bacterial culture and manipulation

2.6.1 Bacterial strains

Table 4: Bacterial strains

TOP10	*F- mrcA Δ(mrr-hsdRMSmcrBC) Φ80dlacZM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG*
DH10B	*F^- mcrA Δ(mrr-hsdRMSmcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu) 7697 araD139 galU galK nupG rpsL λ^-*

2.6.2 Culture media and selective drugs for bacteria

Table 5: Culture Media and selective drugs for bacteria

LB-medium	10g/l Bacto-Trypton 10g/l Bacto-Yeast-extract 5g/l NaCl
Agar plates	15g/l agar in LB-medium
Ampicilin	Stock solution: 100mg/ml in ethanol Final concentration media: 100μg/ml Final concentration agar plates: 100μg/ml
Chloramphenicol	Stock solution: 5mg/ml in ethanol Final concentration media: 2.5μg/ml Final concentration agar plates: 12.5 μg/ml
Kanamycin	Stock solution: 5mg/ml in H_2O Final concentration media: 2.5μg/ml Final concentration agar plates: 12.5 μg/ml
Tetracycline	Stock solution: 20mg/ml in ethanol Final concentration plates: 5μg/ml Final concentration agar plates: 10μg/ml

2.6.3 Preparation of electrocompetent bacteria

5ml of an overnight culture were added to 1L LB-media and cells were grown shaking at 37°C until an OD_{600} of 0.5 was reached. Cells were centrifuged for 10 minutes at 3000 rpm at 4°C. The cell pellet was resuspended in 1L ice-cold water and centrifuged again. Subsequently the pellet was resuspended in 500ml ice-cold water and centrifuged once again. Then, the pellet was resuspended in 20ml ice-cold water containing 10% glycerol (v/v). The suspension was centrifuged at 3500rpm for 15 minutes at 4°C. The cell pellet was resuspended in 2ml water containing 10% glycerol (v/v). Cells were divided into 50μl aliquots, shock frozen with liquid nitrogen and stored at- 70°C.

2.6.4 Transformation of DNA into electrocompetent *E.coli*

A 50µl aliquot of electrocompetent bacteria was thawed on ice and mixed with 1µl of DNA. The mixture was transferred into a sterile pre-cooled 0.2cm electroporation cuvette and exposed to an electric field. Pulse controller and gene pulser were set at 2.5kV, 25µF and 200Ω. The time constant should range between 4-5ms. Immediately after transformation 1ml of LB-media was added to the cells in the cuvette and bacteria were shaken for 1h at 37°C. Afterwards the cells were plated in different dilutions on agar plates containing the appropriate antibiotic.

2.6.5 Transformation of DNA into chemical competent *E.coli*

A 50µl aliquot of chemical competent bacteria was thawed on ice and mixed with 1µl of DNA. Cells and DNA were incubated for 2 minutes on ice. Afterwards cells were incubated for 90 seconds at 42°C. Immediately afterwards cells were once again incubated for 2 minutes on ice. Bacteria were transferred to 1ml LB-media without antibiotics and incubated for 1h shaking at 37°C. Later on, cells were plated in percentage of 10% and 90% on LB-agar plates containing the appropriate antibiotics. Plates were incubated at 37°C overnight.

2.6.6 Preservation of bacterial strains

For long-term storage bacteria suspension were mixed with 87% glycerol in a 1:1 ration and stored at -20°C or -70°C.

2.7 Culture and manipulation of eukaryotic cells

2.7.1 Cell lines

Table 6: Eukaryotic cells lines

NIH/3T3	Embryonic mouse fibroblasts (MEF) cell line, ATCC®CRL 1658
R1-E	Mouse embryonic stem cell line, ATCC® SCRC-1036

2.7.2 Cell culture media and solutions

Table 7: Culture Media and components for eukaryotic cells

Dulbecco´s Modified Eagle´s Medium (DMEM)	13.63g/l DMEM powder (Sigma) 44mM $NaHCO_3$, 10mM HEPES, pH7.2
Dulbecco´s Modified Eagles´s Medium (DMEM) + GlutaMAX-I	Gibco
TEP (Trypsin-EDTA)	6mM EDTA, 0.1% Trypsin (Gibco) in PBS
100X Pen/Strep	6.06mg/ml ampicillin (10.000U/ml) 10mg/ml streptomycin pH7.4
100X Glutamine	29.23mg/ml Glutamine
Gelatine solution	2% stock solution type B from bovine skin, diluted to 0.1% (w/v) with PBS
Fetal Calf Serum (FCS)	Biochrome (for somatic cells) Sigma F7524 for ES cells
Leukaemia Inhibitory Factor (LIF)	ESGRO $10X10^6$U/ml
100X Non-essential Amino Acids	Gibco
100X β-Mercaptoethanol	Gibco
G418	100mg/ml G418 in ddH_2O
Doxycycline	2mg/ml doxycycline-hyclate (Sigma) in 70% ethanol
100mM Sodium Pyruvate	Gibco
PBS	140mM NaCl, 27mM KCl, 7.2mM Na_2HPO_4, 14.7mM KH_2PO_4, pH6.8-7.0
Hygromycin B	Calbiochem
Mitomycin C	Fisher scientific (2mg)

2.7.3 Media composition

Table 8: Media composition for eukaryotic culture media

DMEM3+	DMEM 1X Pen/Strep 1X Glutamine 10%FCS
DMEM5+	DMEM 1X Pen/Strep 1X Glutamine 10%FCS 1X Non-essential Amino Acids 1X β-Mercaptoethanol
ES Media	DMEM-GlutaMAX-I 1X Pen/Strep 1X Sodium Pyruvate 1X Non-essential Amino Acids 1X β-Mercaptoethanol 60µl LIF 15%FCS (heat-inactivated for 30 minutes at 56°C)

2.7.4 Cultivation of eukaryotic cells

Cell were cultivated in flasks or plates at 37°C and 5%CO_2 and 21%O_2 at maximal humidity. Cell culture media was exchange every 3-4 days and dependent on confluency cell were divided into new culture flasks or plates. For dividing cell culture, media was removed and cells were washed once with pre-warmed PBS. Subsequently cells were trypsinized until cells detached from the plastic surface. Trypsin inactivation was performed by addition of FCS containing media. Cells were centrifuged for 5 minutes at 3000rpm and resuspended in fresh media. Afterwards an aliquot was transferred into a new culture flask or plate containing fresh media. For determination of cell number cells were counted automatically using Guava EasyCyte Flow Cytometer (Milipore) or manually using a Neubauer counting chamber.

mES cells were cultivated on gelatinized feeder covered tissue flasks in order to maintain pluripotency of the cells. Gelatinized flask were prepared by addition of Gelatine solution. The flasks were incubated for 15 minutes at 37°C and the residual gelatin solution was removed. Afterwards mitotically inactivated feeder cells were seeded into the flask. One day after feeder seeding flask were used for maintenance of mES cells.

mES cells were cultivated at 37°C, 7%CO_2 and 2%O_2 at maximal humidity. Media change was performed daily and subcultivation was performed every second day to avoid differentiation and to keep them under constant proliferation. The media was removed and mES cells were washed twice with pre-warmed PBS. Afterwards trypsin was added and cells were kept at 37°C for 5 minutes until FCS containing media was added. The cell suspension was centrifuged for 5 minutes at 3000 rpm and the cell pellet was resuspended in fresh media and an aliquot was subcultivated into new gelatinized feeder covered culture flasks.

Table 9: Cell line and media composition

Cell line	**Media composition**
NIH3T3	DMEM3+
R1-E	ES Media
DIMx2-tTA	DMEM5+

2.7.5 Stimulation of cells with IFN-β

IFN-β was produced in-house using supernatants of stable-transfected BHK-1 cells. The activity of IFN-β was determined on Mx2Luc2 cells (Pulverer et al., 2010) and related to the activity of recombinant IFN-β of known concentration.

For IFN-β stimulation experiments cells were treated with indicated IFN-β concentrations (mainly 500U/ml). Subsequently cells were analysed either 24hrs later or at indicated time-points.

2.7.6 Mitomycin treatment

2mg of Mitomycin C were dissolved in 5ml PBS and the Mitomycin/PBS solution was transferred into 195ml 5+ Media to have a final concentration of 10µg/ml Mitomycin C. The Mitomycin C containing media was sterilized using a 60µm Nylon Net Filter and aliquots were prepared. These aliquots can be stored at -20°C.

One day before addition of selection media feeder cells were treated with Mitomycin C containing media. Therefore the media supernatant was removed and the Mitomycin C-Media solution was added onto the cells and incubate for 2-3h at at 37°C in the incubator. Afterwards the Mitomycin-Media was removed and the cells were washed once with PBS. Cells were cultivated after treatment in 5+ media.

The Mitomycin treatment inhibts feeder proliferation and is an alternative to irradiation methods. The Mitomycin-Media solution has to be inactivated using UV-light before it can be discarded.

2.7.7 Gene transfer methods

Transfection was performed using Lipofectamine®2000 from Invitrogen. Liposomal transfection was performed according to the manual from Invitrogen. In brief, one day prior transfection cells were seeded to reach 70-80% confluency the next day. The respective amount of DNA and Lipofectamine®2000 was mixed in serum-free media and incubated for 20 minutes at room temperature. Cells were washed once with PBS and the mixture was transferred to the cells. After 5 hours FCS containing media was added to the cells. For transient expression experiments cells were analyzed 48 hours post transfection.

2.7.8 Isolation of clones after selection pressure

After two days post transfection cells were transferred from a 6-well plate format to a 10cm dish in media containing the appropriate concentration of selection reagent. The media was daily changed until single colonies grew to an appropriate size. Cells were washed with PBS once and kept in PBS while single colonies were isolated and transferred into a 96-well plate using a microscope. Cells were expanded and analyzed.

Table 10: Transgenic cell lines and selection conditions

	NIH/3T3 (BAC-Mx2-tTA)	ESMx2-tTA / DIMx2-tTA
G418	1mg/ml	0.8mg/ml
Hygromycin B	300U/ml	300U/ml

2.7.9 Kryoconservation of eukaryotic cells

Cells in the exponential growth phase were washed once with PBS and detached from the culture flask or plate using TEP. Afterwards cells were centrifuged for 5 minutes at 3000rpm. The cell pellet was resuspended in FCS containing 5%DMSO and transferred to kryo-vials. Vials were stored at -70°C in an isopropanol for approximately 24 hours and afterwards transferred to liquid nitrogen for long term storage.

2.8 Isolation and preparation of DNA from *E.coli*

2.8.1 Small Scale Isolation of Plasmid DNA

STET buffer	80g/l Sucrose 0.5% Triton X100 50mM EDTA 10mM Tris/HCl pH8.0
TE-buffer	0.1mM EDTA 10mM Tris/HCl pH8.0
Lysozyme	10mg/ml Lysozyme in TE buffer
Ammonium acetate	8M NH_4OAc
TE RNase	10μg/ml RNase A in TE buffer

A 2ml LB medium culture containing the appropriate antibiotic was inoculated with a single colony and cultured shaking over night at 37°C. The next day the suspension was centrifuged at 13.000rpm for 1 minutes and the pellet was resuspended in 500μl STET buffer. For bacterial cell lysis 50μl lysozyme solution was added and incubated at room temperature for maximal 3 minutes. Heat inactivation was performed at 95°C for 90 seconds. Afterwards the suspension

was centrifuged at 13.000rpm for 5 minutes. Bacterial chromosomal DNA was removed and plasmid DNA was precipitated with 50µl 8M NH_4OAc and 0.5ml isopropanol. The samples were centrifuged at 13.000rpm for 5 minutes and the supernatant was removed. Plasmid DNA was dried at room temperature and dissolved in 50µl TE buffer.

2.8.2 Large Scale Isolation of plasmid DNA

Maxi preparation (Qiagen or Macherey Nagel) was performed according to the manufacturer´s instructions. In brief 300ml LB media containing the appropriate antibiotic was cultured while shaking over night at 37°C. Bacteria were centrifuged for 20 minutes at 5000rpm and lysed afterwards. The reaction was neutralized and transferred to plasmid DNA binding columns. After several washing steps plasmid DNA was diluted and precipitated with isopropanol. The suspension was centrifuged at 14.000rpm for 15 minutes and plasmid DNA was washed with 70% ethanol and centrifuged again at 14.000rpm for 5 minutes. The DNA pellet was air dried and dissolved in an appropriate amount of TE buffer.

2.8.3 Large scale isolation of BAC DNA with BAC Maxi Prep (BAC 100)

Bacteria were cultivated in 500ml LB with the appropriate antibiotic at 37°C overnight. Cells were harvested at 5000rpm for 20 minutes at 4°C and isolation of BAC DNA was performed according the manufacturer´s instruction. DNA was re-suspended in 100µl TE+RNase and thoroughly shaked at 100rpm at 37°C for 1 hours. Resuspension steps were performed with truncated pipette tips to reduce shearing of the BAC DNA.

2.9 Isolation and preparation of nucleic acids from eukaryotic cells

2.9.1 Isolation of high molecular weight DNA

Modified Bradley´s solution	10mM Tris/HCl pH7.5 2mM EDTA 10mM NaCl 0.5% SDS
Proteinase K	1mg/ml
Ethanol/Sodium Acetate	96% ethanol 75mM NaAc

Cells were trypsinized and centrifuged at 3000rpm for 3 minutes. The pellet was washed once with PBS and centrifuged once again. The pellet was then resuspended in 500µl Bradley´s containing 0.05mg/ml Proteinase K. The mixture was incubated slightly shaking for 30 minutes. Samples were cooled to room-temperature and then 1ml of 96% ethanol containing 75mM NaAc was added. The samples was turned around several times until precipitated chromosomal DNA became visible. The mixture was centrifuged at 5000rpm for 5 minutes and washed with 0.5ml 70% ethanol and afterward centrifuged once again. The pellet was air drying and resuspended in 50µl TE-buffer. Finally, DNA samples were incubated at 37°C until DNA was dissolved.

2.9.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to its size. As not elsewhere mentioned 1% agarose gels were prepared by suspending 1g agarose into 100ml 1XTAE buffer. The solution was boiled until the agarose dissolved. The solution was poured into a trail and 2µl Midori Green were added and mixed. After the gel solidified it was transferred into an electrophoresis chamber and submerged in 1XTAE buffer. DNA samples were mixed with 5X loading buffer and applied onto the gel. To determine fragment size of the separated DNA a marker was loaded in parallel (Hyperladder I, Bioline). Electrophoresis was performed at 100V and gels were examined under UV-light (360nm).

2.9.3 Purification of DNA

DNA fragments from restriction reactions were purified by agarose gel electrophoresis and subsequent isolation from the gel. This is normally performed when additional fragments are present in the mixture so that separation is needed. Fragments from gels were purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer´s manuals
PCR reaction with a single specific product were purified with the QIAquick PCR purification kit (Qiagen) following the manufacturer´s instructions.

2.10 Modification of DNA

2.10.1 Restriction digestion of DNA

For DNA restriction by endonucleases reaction conditions (buffer, temperature) for each enzyme were used as proposed by the manufacturer (NEB, Roche). Reactions were stopped either by heat inactivation or column purification.

2.10.2 Fill in reactions of 5´ primer overhangs

1µg of DNA with 5´ primer overhangs was mixed with 3µl 10mM dNTP, 1X Klenow buffer and 1U Klenow enzyme in a total volume of 50µl. Incubation was performed for one hour at 37°C and afterwards purified using the QIAquick PCR purification kit.

2.10.3 Dephosphorylation of DNA fragments

To avoid religation after endonuclease digestion compatible overhangs were dephosphorylated using 1U CIP. The enzyme was directly applied in the previous used reaction mixture and incubated 30 minutes at 37°C. Afterwards the reaction was stopped by column purification.

2.10.4 Ligation reaction

Ligation of two compatible DNA fragments was performed in a 1:4 ratio between backbone:insert. Therefore 1U T4 DNA ligase was mixed with DNA fragments and 1Xligase buffer in a total volume of 10µl and incubated overnight at room temperature.

2.10.5 Mutagenesis of Bacterial Artificial Chromosome vectors (BACs)

The BAC RP24-71I6 from BacPac Resource Center (http://chori.org/BACPAC) from the RPCI-23/24 Rosewell Park Cancer Institute Library used before for generation of BAC-Mx2-Luc (Pulverer et al., 2010) was used for BAC mutagenesis . First, DH10B bacteria were established carrying the BAC RP24-71I6 via electroporation. A single bacteria clone was isolated on the basis of Chloramphenicol resistance encoded on the BAC. Subsequently this DH10B carrying the BAC were transformed via electroporation with the pKD46 plasmid. This plasmid encodes for the λ-phage recombination enzymes, redα, redβ and redγ and has a temperature sensitive origin of replication. Replication of the plasmid in DH10B bacteria was permissive at a temperature of 30°C while the plasmid was lost when bacteria were cultivated at a temperature of 43°C. Thus, *E.coli* containing the BAC RP24-71I6 and the pKD46 were incubated at 30° on agar plates with ampicillin (pKD46) and chloramphenicol (BAC RP24-71I6). Expression of the λ-recombination enzymes was induced by addition of 1% arabinose to a suspension culture within their exponential growth phase. Bacteria were grown to an OD_{600} of 0.5-0.8 and made electrocompetent.
The plasmid pUHD15-1-Mx2HR-tTA was digested with BsBI generating a fragment of 5369bp. This was transformed via electroporation into DH10B containing pKD56, the BAC RP24-71I6 and the λ-recombination enzymes to perform *in vitro* recombineering. Selection was performed on agar plates containing Chloramphenicol (BAC RP24-71I6) and Kanamycin (selection marker on recombination fragment) at 43°C to eliminate pKD46 plasmid. Loss of pKD46 was tested via ampicillin sensitivity. For analysis of homologous recombination recombined BACs were subjected to colony PCR.

2.11 Analysis of nucleic acids

2.11.1 Polymerase chain reaction (PCR)

For standard and colony PCR MasterMix from p.j.k was used whereas the Expand™ Long Template PCR system was used for cloning purposes. Oligonucleotides were purchased from MWG with a GC content of 50% and a CG clamp at the 3´ prime end. It was verified that oligonucleotides used within the same PCR reaction share a similar estimated melting

temperature. Table 9 and 10 summarizes the composition of the individual PCR reaction setups.

Table 11: PCR reaction mix for standard and colony PCR

2X PCR Master Mix	15µl
10pmol/ µl forward primer	1 µl
10pmol/ µl reverse primer	1 µl
DMSO	1 µl
DNA template	100ng
H_2O	ad 30 µl

Table 12: PCR reaction mix for Expand™ Long Template PCR

5X reaction buffer	10 µl
10pmol/ µl forward primer	1 µl
10pmol/ µl reverse primer	1 µl
10mM dNTP Mix	4 µl
DMSO	1 µl
DNA polymerase (2.5U)	0.5 µl
DNA template	100ng
H_2O	Ad 50 µl

Dependent on the oligonucleotide combination used the annealing temperature was adjusted. The same is true for the elongation time of the fragment which should be obtained. Importantly the polymerase in the 2XPCR Master Mix has an optimal elongation temperature of 68°C whereas the polymerase from the Expand™ Long Template PCR system has an optimum at 72°C. Table 11 summarizes a general PCR programme.

Table 13: General PCR programm

Expand Long Template Polymerase		
Step	Temperature (°C)	Time (min)
1	95	5
2*	95	0.5
3	58-63	0.5
4	72	0.5/kb
5	72	10
6	16	hold

BIOMIX		
Step	Temperature (°C)	Time (min)
1	95	5
2*	95	0.5
3	58-63	0.5
4	68	1/kb
5	68	10
6	16	hold

*steps 2-4 were repeated 30 times

For colony PCR the BIOMIX was used and MasterMixes were prepared according to Table 11 without addition of DNA. A single bacteria colony was picked with a toothpick or a pipette tip and transferred on a Master Plate containing the appropriate antibiotics and afterwards into the tube containing the PCR reaction mix. Samples were subjected to PCR analysis and fragments were separated on a 1% agarose gel.

Oligonucleotides were chosen on the basis of their binding properties. One oligonucleotide was chosen to bind to the vector backbone while the other oligonucleotide was chosen to be specific for the integrated DNA insert.

2.11.2 Quantitative real time PCR analysis

2.11.2.1 Isolation of total RNA from eukaryotic cells

RNA was isolated with the RNeasy Mini Kit from Qiagen according to manufacturer´s instructions. Cultivated cells were harvested and homogenized in presence of 600µl of a highly denaturating guanidine isothiocyanate-containing buffer inactivating RNases to ensure isolation of intact RNA. Then 600µl of 70% ethanol was added to provide appropriate on column binding conditions. Maximal 700µl of this solution was loaded on a RNeasy mini column where total RNA bound to the membrane. On column DNA digestion was performed additionally. After several washing steps RNA was eluted in 50µl RNase free water.

2.11.2.2 cDNA synthesis

Total mRNA was reverse transcribed using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer instruction. In brief 2μg of RNA were diluted in 11μl of water and heated at 65°C for 5 minutes. Afterwards samples were cooled on ice for 2 minutes. Subsequently, 9μl of cDNA Master Mix was added to each sample (including 0.5μl oligo dT primers). Samples were incubated at 45°C for 1hour. Heat-inactivation of reverse transcriptase was performed for 5 minutes at 72°C.

2.11.2.3 qRT-PCR

Quantitative Real-time PCR was performed on the LightCycler® (Roche) in a total volume of 20μl using the SybrGreen Mix from Qiagen. Expression of the target gene was normalized to expression of β-actin as a housekeeping reference gene. cDNA samples were diluted 1:4 before addition to the PCR reaction.

Table 14: MasterMix for qRT-PCR

SybrGreen	10μl
Forward primer	1μl
Reverse primer	1μl
DNA template	8μl

Table 15: Programm for qRT-PCR

Step	Temperature (°C)	Time (mm:ss)
1	95	5
2*	95	0:10
3	58	0:20
4	72	0:30

*steps 2-4 were repeated 45X

At the end of the RT-PCR run a melting curve was performed. The relative expression of specific genes was determined in relation to the expression of β-actin. Based on the equation $2^{-\Delta Ct}$. The ΔCt value is obtained by the difference between the ct value of the target and the reference gene.

2.12 *In vivo* experiments

2.12.1 Transplantation of transgenic cells

For allotransplantation immunocompromised *RAG2/IL2rgc* double knockout mice were used and the back of the mice was shaved with an electric razor prior transplantation (Mazurier et al., 1999). Transgenic cells were trypsinized and centrifuged at 1200rpm for 5 minutes. Cells were washed and resuspended in PBS (without Ca^{2+} and Mg^{2+}) so that a cell number of $1X10^7$cells/ml was obtained. Cells were transported on ice into the mouse facility and 0.1ml of the cell suspension ($1X10^6$ cells) was subcutaneous injected into the back of the mice while these were under isoflurane anesthesia.

2.12.2 Treatment with polyI:C

For analysis of *in vivo* IFN response by transplanted cells, mice were treated intraperitoneally with 200μg polyI:C (diluted in 100μl PBS) one day after cell transplantation.

2.12.3 Bioluminescence Imaging with Xenogen IVIS 200

For obtaining *in vivo* images animals were anaesthetised in the induction chamber by 2-2.5% isoflurane (Abbot) first. The mice were injected with 100µl of luciferin (30mg/ml in PBS, Synchem OHG) and put on the heated (37°C) platform in the acquisition chamber. Anaesthesia was maintained throughout the whole procedure by constant administration of isoflurane via nose cones. After an incubation time of 10 minutes images were taken. Analysis of the images were performed with the Living image 2.60.1 (Igor Pro4.09A) software.

2.13 Vectors and oligonucleotides

2.13.1 Applied vectors

Table 16: Applied vectors with general information used in this study

pDHC003	Also known as pLV-hU6.Cas9GFP. Cloning vector for integrating BsmBI flanked oligonucleotides to generate gRNAs. This plasmid was provided by Dirk Heckl (MHH – Hannover); N-Number 4314
pHD15-1	Expression vector encoding for tTA transactivator under control of a cytomegalovirus promoter (Gossen and Bujard, 1992); N-Number 945
pGL4.10FPGKNeo	Vector containing a dual promoter fragment driving expression of the kanamycin/neomycin phosphotransferase resistence flanked by flippase recognition target sites (Promega, Pulverer et al., 2010); N-Number 3762
RP24-71I6	Bacterial artificial chromosome contains genomic sequence (C57BL/6J) 97540510-97790829bp on Chromosome 6; harbours Mx1 and Mx2 genes (BACPAC www.bacpac.chori.org; Pulverer et al., 2010); N-Number 3027
pCR4-TOPO-mIL10	The cloning vector contains the coding sequence of murine IL10 and was a gift from Prof. Müller Newen (Aachen); N-Number 4524
pTRE-tight	Cloning vector with tTA promoter (Clontech); N-Number 3891
pHT	Expression vector encoding for a fusion protein of hygromycin and thymidine kinase under the control of the SV40 promoter. (Frank Kuhnert, Diploma thesis; N-Number 171)
pRBT1Luc	Expression vector encoding for firefly luciferase under the control of a bidirectional tTA promoter (J. Unsinger; N-Number 1951)

The N-Number refers to an internal database. where additional more detailed

2.13.2 Cloned vectors

Table 17:Vectors generated during this study with cloning strategy

pDHC003-gRNA+40	E2 gRNA MX2_gRNA+40_for and MX2_gRNA+40_rev were annealed and ligated into pDHC00 digested with BsmBI; N-Number 4501
pDHC003-gRNA+40E14	E14.1 gRNA Mx2-gRNA+40E14_for and Mx2-gRNA+40E14_rev were annealed and ligated into pDHC003 digested with BsmBI; N-Number 4502
pDHC003-gRNA+41E14	E14.2 gRNA Mx2-gRNA+41E14_for and Mx2-gRNA+41E14_rev were annealed and ligated into pDHC003 digested with BsmBI; N-Number 4504
pDHC003-gRNA+54E14	E14.3 gRNA Mx2-gRNA+54E14_for and Mx2-gRNA+54E14_rev were annealed and ligated into pDHC003 digested with BsmBI; N-Number 4503
pHD15-1-5hom-Mx2	Subcloning vector 1000nt fragment of the 5´untranslated region in front of the ATG of Mx2 was amplified with X-Mx2.5-for and E-Mx2.5-1-rev from genomic DNA and ligated in pHD15-1 using XhoI and EcoRI restriction sites; N-Number 4459
pHD15-1-5hom-Mx2-3hom	Subcloning vector 1000nt fragment of the 3´untranslated region after TAA of Mx2 was amplified with H-Mx2.3-for and H-Mx2.3-1-rev from genomic DNA and ligated into pHD15-1-5hom-Mx2 using HindIII restriction sites; N-Number 4505

pHD15-1-Mx2HR-tTA	Homology template containing selection cassette pHD15-1-5hom-Mx2-3hom was linearized using NaeI, Klenow-Fill in reaction was performed and vector backbone was dephosphorylated. pGL4.10FPGKNeo was digested with BamH1. After Klenow-Fill in reaction the 2175bp fragment was ligated into linearized pHD15-1-5hom-Mx2-3hom backbone; N-Number 4500
pTREtight-mIL10	Transactivator driven mIL10 expression construct mIL10 was amplified from pCR-TOPO-mIL10 using Xma-Kozak-mIL10-for and EcoRV-mIL10-rev and ligated intp pTREtight using Xma and EcoRV restriction sites; N-Number 4525

2.13.3 Engineered Bacterial artificial chromosome vectors

Table 18: BAC generated in this study with cloning strategy

BAC-Mx2-tTA	The repair template pHD15-1-Mx2HR-tTA was digested with BsBI and the 5369bp fragment was inserted into RP24-71I6 by recombineering; N-Number 4521

2.13.4 Oligonucleotides for cloning

Table 19: Oligonucleotides used in this study for cloning

Name	**5´-3´ Sequence**
X-Mx2.5-for	CCCTCGAGCCATGTTCTTGTTTCCAACTGGGCTGAGG
E-Mx2.5-1-rev	CCGAATTCCTTTTATTTCTTCTCTCAGTTGGG
H-Mx2.3-for	CCAAGCTTGCCTCAAAAGCGCCTTGCTCTC
H-Mx2.3-1-rev	CCAAGCTTCTCTGCTGGCTGGCTGGTGGC
Mx2-196hom-for	GGGGACATGGAGGACAGGAA
tTA-TGA-rev	CCCACCGTACTCGTCAATTC
Neo-for	CCTGAATGAACTGCAGGACG
Mx2-104hom-rev	GGGGTGCTGGCTCATCAGAT
sgRNA+263_SA-for	CCCTGGTTGAGCCAGAGGGC
sgRNA+263_SA-rev	CGGCCCCAACACTTGGAGTGG
Xma-Kozak-mIL10-for	GGCCCGGGCACCATGCCTGGCTCAGCACTGCT
EcoRV-mIL10-rev	GGGATATCTTAGCTTTTCATTTTGATCA
Mx2-gRNA+54E14-for	CACCGATTGGGGGAAGGGTTTGCAC
MX2-gRNA+54E14rev	AAACGTGCAAACCCTTCCCCCAATC
Mx2-gRNA+41E14-for	CACCGACTCACTTTGAATTGGGGGA
Mx2-gRNA+41E14-rev	AAACTCCCCCAATTCAAAGTGAGTC
Mx2-gRNA+40E14-for	CACCGTTTGACTCACTTTGAATTGG
MX2gRNA+40E14-rev	AAACCCAATTCAAAGTGAGTCAAAC
MX2_gRNA+40_for	CACCGAACTTACCCAGTGGAGAGAC
MX2_gRNA+40_rev	AAACGTCTCTCCACTGGGTAAGTTC

2.13.4 Oligonucleotides for qRT-PCR

Table 20: Oligonucleotides used for qRT-PCR

Name	**5´-3´ Sequence**
tTA-1-for	GGACGAGCTCCACTTAGACG
tTA-1-rev	AGGGCATCGGTAAACATCTG
MX2.1RT-for	TCACCAGAGTGCAAGTGAGG
MX2.1RT-rev	CATTCTCCCTCTGCCACATT
mIL10.1RT-for	CCAAGCCTTATCGGAAATGA
mIL10.1RT-rev	TTTTCACAGGGGAGAAATCG
Actin for	TGGAATCCTGTGGCATCCATGAAAC
Actin rev	TAAAACGCAGCTCAGTAACAGTCCG

3. Results

3.1. Development of a system to rewire infection related signals to synthetic cassettes

3.1.1. Overall strategy and design of the expression cassettes

To rewire infection related signals a novel modular synthetic circuit was established. This circuit is composed of the murine Mx2 promoter as a prototype promoter of IFN stimulated genes that has been shown to be specifically activated during viral and bacterial infections (Pulverer et al., 2010) as well as under conditions of sterile inflammation (Rais et al., 2016). Therefore this promoter was chosen in purpose of rewiring physiological infection related signals. In order to connect the IFN input signals to synthetic cassettes the tetracycline transactivator (tTA) of the Tet-off system was used as a synthetic transcription factor to specifically induce expression of target genes of interest. In the presence of IFN the cognate receptor mediated signalling activates the Mx2 promoter and thereby results in expression of the tTA transactivator. This in turn can activate expression of genes of interest which are controlled via the Tet-promoter (Figure 4).

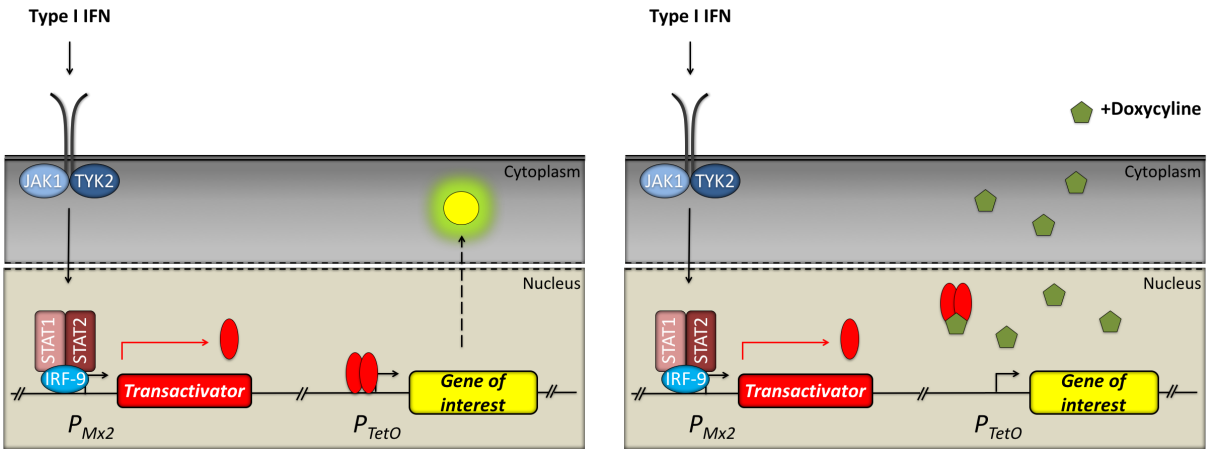

Figure 4: Schematic representation of synthetic circuit established to link physiological type I IFN expression to synthetic expression cassettes.

Binding of IFN to its cognate receptor induces the Jak-STAT pathway leading to the expression of the synthetic transactivator tTA from the Mx2 promoter (P_{Mx2}). Upon binding of tTA to its cognate DNA binding regions (P_{TetO}) transgene expression is induced (left scheme). In the presence of doxycycline the ability of the tTA transactivator for DNA binding is impeded resulting in absence of transgene expression (right scheme).

Due to the modular character of the synthetic circuit various cassettes can be integrated. This offers a kind of flexibility and the opportunity to design the circuit for a particular/specific biological scenario.

Since the binding capacity of the tTA is controlled by tetracycline or its derivatives such as doxycycline, in presence of doxycycline gene expression from *tetO* sequences will be repressed (Figure 4). This provides the possibility to abrogate transcription of the gene of interest even in the presence of the physiological IFN stimulus.

Previously, it was shown that the Mx2 promoter is controlled by proximal as well as remote elements (Pulverer et al., 2010). Thus, to ensure that all regulatory elements needed for the tight regulation are maintained, the tTA transactivator was targeted into the cellular Mx2 locus by homologous recombination in this study. Two different strategies were followed to achieve targeting. In the following chapter, the targeted integration of the tTA into the endogenous chromosomal Mx2 locus of murine embryonic stem cells (mES) is described. In chapter 3.5 *in vitro* recombineering is described that was used to integrate the transactivator into a bacterial artificial chromosome (BAC) vector comprising 150kb of the mouse Mx2 locus.

3.1.2 CRISPR-Cas9 mediated homologous recombination of the synthetic doxycycline dependent tTA transactivator into the murine Mx2 locus

To integrate the synthetic transactivator tTA into the Mx2 locus the CRISPR/Cas9 technology was used as a tool to induce double strand breaks and thereby enforce targeted integration via homologous recombination. To do so the tTA gene was flanked with about 1kb of the 5´ and 3´ sequences that are homologous to the endogenous Mx2 locus (Figure 5). The homology sequences compromise parts of exon 2 and exon 14 respectively, thereby restoring the putative regulatory sequences up- and downstream and eliminating the Mx2 coding sequence upon homology dependent recombination.

Furthermore, to facilitate selection the neomycin resistance gene was included and controlled by the internal constitutive PGK promoter.

The strategy for homologous recombination was designed in a way that after integration, the ATG initiation codon of the endogenous Mx2 gene would be the start codon of the tTA transactivator. To achieve efficient recombination, various Cas9 target sequences were chosen that are located close to the homologous regions within the coding sequence of Mx2 but not within the target vector. This was done to make sure that the targeting vector cannot serve as a template for Cas9 activity (Figure 5).

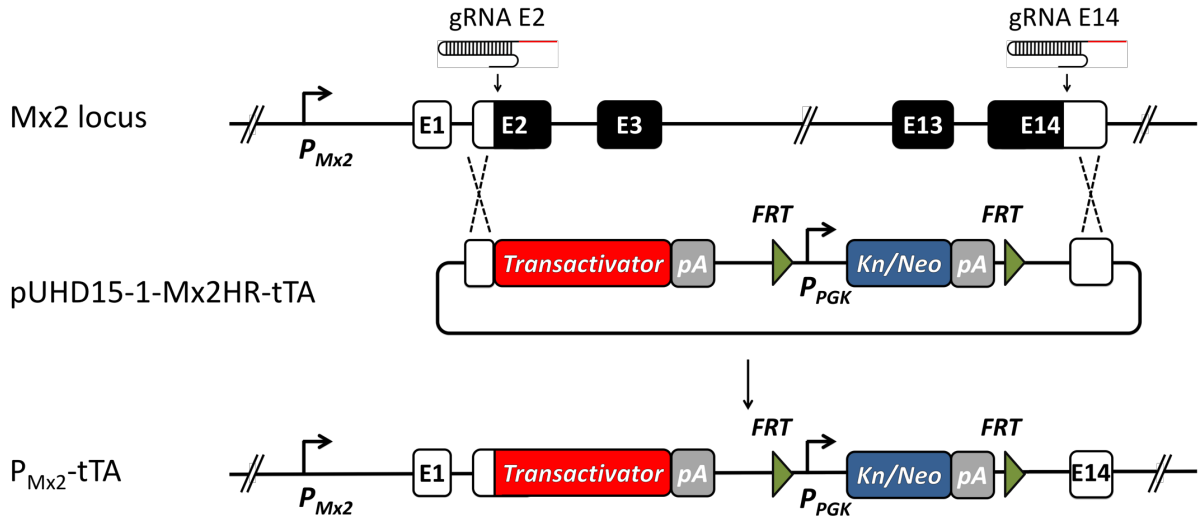

Figure 5: Homologous recombination strategy initiated by Cas9 induced DNA double strand breaks.

DNA double strand breaks were induced by targeting the RNA-guided DNA endonuclease Cas9 to specific sequences within the Mx2 locus using gRNAs. These are indicated as gRNAE2 specific for sequences within exon 2 and gRNAE14 specific for sequences within exon 14, respectively. DNA double strand breaks induce the DNA repair pathway and foster homologous recombination. For efficient and site specific integration the template plasmid (pUHD15-1-Mx2HR-tTA) contains 5´ and 3´ sequences (indicated as white rectangles) that are homologous to the Mx2 locus. For selection purposes the template contains a kanamycin/neomycin selection cassette driven by a constitutive PGK promoter. The cassette is flanked by FRT sites which provides the option to excise the cassette by flp recombinase. Note that the schematic drawing is not scaled.

In order to select optimal Cas9 target sites and derive gRNA sequences the algorithm from the Zhang lab was used (http://crispr.mit.edu/). This algorithm searches for possible Cas9 target sequences which consist of 20 nucleotides followed by a protospacer adjacent motif (PAM). In this study Cas9 from *Streptococcus pyogenes* was used which relies on the canonical PAM sequence 5´-NGG-3´. PAM is an essential targeting compound because Cas9 will not cut if the PAM is missing. A sequences of about 250nt in close proximity to the start or stop codons of Mx2, respectively, were screened for suitable PAM sites by the algorithm. Using this algorithm, a score is provided for each binding motif. This score integrates and quantifies the contribution of mismatch locations, density and identity on Cas9 on-target and off-target cleavage. Upon applying this tool, there was only one suitable gRNA targeting sequence within exon 2 fulfilling the following criteria: a) a close proximity to the Mx2 ATG should be given, b) localization of the target sequence downstream to the start ATG and c) a score of greater than 50% as recommended by the websites guide selection guidelines. With regard to the gRNA located in exon 14 three different sequences were considered to be candidate target sequences based on the mentioned criteria. The details for 4 gRNA target motifs used in this study are summerized in Table 21.

Table 21: gRNA binding sequences, location and score values

Name	gRNA target sequence + **PAM**	Target sequence location	On-target locus	Score for gRNA	Off-target Score
E2	AACTTACCCAGTGGAGAGAC**TGG**	Exon2	Chr.16:+97760294	69	3.0
E14.1	TTTGACTCACTTTGAATTGG**GGG**	Exon14	Chr.16:+97782503	63	1.4
E14.2	ACTCACTTTGAATTTGGGGA**AGG**	Exon14	Chr.16:+97782507	59	1.6
E14.3	ATTGGGGGAAGGGTTTGCAC**AGG**	Exon14	Chr16:+97782518	70	4.5

The 4 different 20 nt binding sequences were fused to a gRNA scaffold sequence responsible for Cas9 binding by inserting corresponding DNA oligonucleotides into the DHC003 plasmid. In this plasmid, transcription of the gRNA is mediated by a U6 promoter. Additionally, this plasmid encodes for SpCas9 endonuclease and eGFP as a bicistronic transcript separated by a 2A sequence. For the 4 target sites 4 gRNA vectors were established. For homologous recombination, the vectors were used in different combinations whereas the vector encoding gRNA E2 was always used.

To achieve CRISPR/Cas9 induced homologous recombination the gRNA vector targeting exon 2 and one of the gRNA vectors targeting exon 14 were transfected in mES cells together with the circular pUHD15-1-Mx2HR-tTA repair template via liposomal transfection using Lipofectamine2000®. Two days after transfection the cells were sorted for the expression of eGFP to enrich cells that have taken up DNA and express Cas9. Since the repair template contains a selection cassette cells were cultivated after sorting in the presence of 0.8 mg/ml G418. This allowed to select cells that have integrated the homology vector. The media was daily exchanged as within all subsequent selection processes for the establishment of stable mES cell lines. After the selection process single cell derived clones were isolated and cultivated as individual clonal populations.

To identify cells that have successfully undergone homologous recombination, samples of individual clonal populations were harvested and genomic DNA was isolated. To verify that targeted integration has happened, the isolated genomic DNA was used as template for polymerase chain reaction. The overall process of generation stable targeted mES cells is summarized in Figure 6.

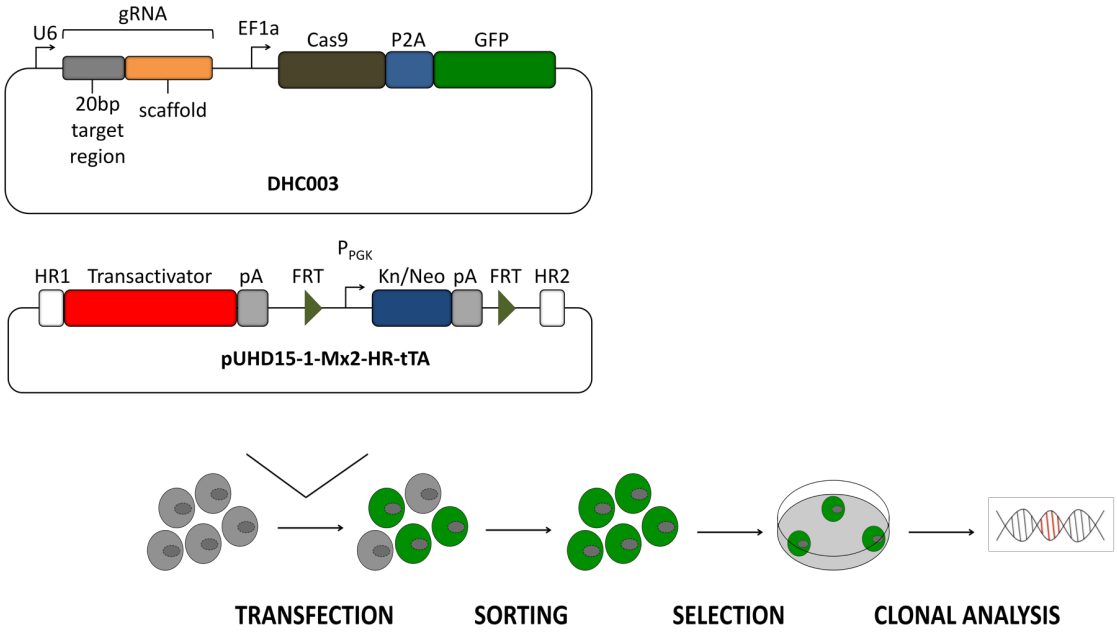

Figure 6: Flow chart for establishment of stable integration of the tTA gene into chromosomal Mx2 locus.

Murine embryonic stem cells were transfected with the pUHD15-1-Mx2HR-tTA repair template encoding the tTA transactivator and the selection cassette flanked by sequences homologous to the Mx2 locus (see figure 2 for details). Along the two DHC003 plasmids were transfected encoding Cas9, eGFP and the gRNAs E2 and one of the gRNAs E14. After transfection the cells were sorted for expression of eGFP via flow cytometry (FACS) to enrich cells for Cas9 expression. Subsequently, these cells were cultivated in the presence of 0.8mg/ml G418 to select for clones that have stably integrated the repair cassette. Individual clones were isolated and screened via PCR for correct targeting of the tTA transactivator in the Mx2 locus.

A frequent side reaction of targeted integration via homologous recombination is random integration of the targeting vector via non-homologous end joining. Also cells that have undergone random integration of the targeting vector would be resistant to G418. In order to distinguish between random and targeted integration a PCR was set up that involved one primer that was located in the genomic DNA and a second primer that was specific for the repair cassette (Figure 7). This was done for both the 5' and the 3' regions of the targeting vector. Polymerase chain reaction was performed on genomic DNA obtained from G418 resistant clones. Amplified PCR products were separated on a 1% agarose gel and stained with Midori Green. Out of 15 tested clones, for 7 clones products could be obtained from both the 5' and the 3' region. The fragments had a size of 2238bp using primer combinations c/d (5') and 2258bp using primer combinations e/f (3'), respectively (Figure 7B) indicating a correct integration of the repair template at the Mx2 locus. Figure 7C shows one representative mES cell clone (ES3-24) which was used for all subsequent studies. Noteworthy, not all G418

resistant clones gave rise to both 5´ and 3´ PCR products. From the 15 tested clones 7 showed neither a 5´ nor a 3´ prime integration although they were resistant to G-418. These clones

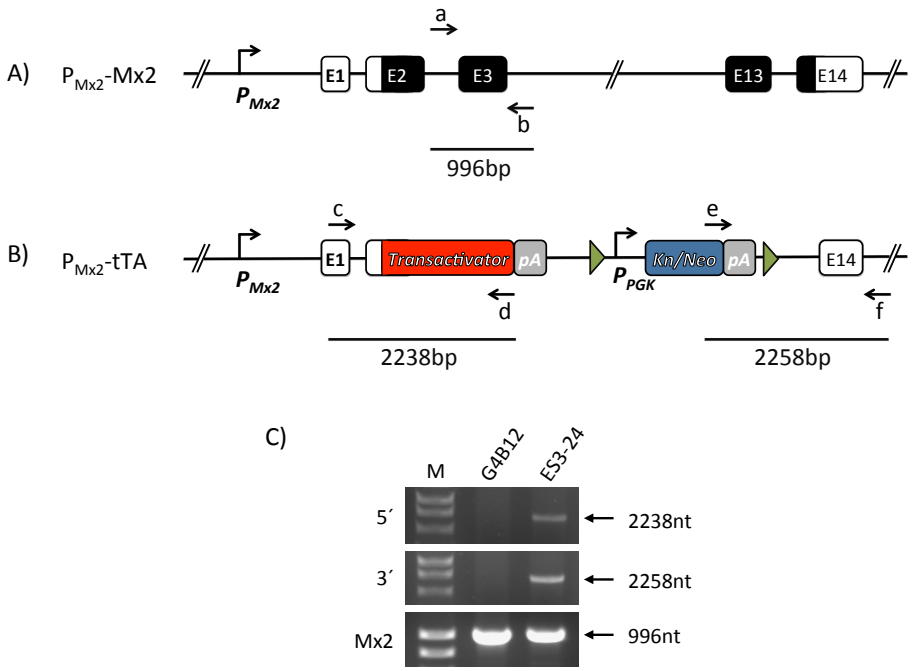

Figure 7: PCR analysis to prove targeted integration of the tTA transactivator in the Mx2 locus.

The G418 resistant clones were analysed for the presence of the transactivator within the targeted Mx2 locus. Genomic DNA was isolated and PCR was performed using specific oligonucleotide combinations. A) Schematic representation of the endogenous Mx2 locus with the coding sequence for the murine Mx2 gene White rectangles indicate intron regions and black rectangles indicate exon regions. Forward and reverse oligonucleotides used for PCR are indicated as black arrows (a on the + and b on the - strand). In presence of the murine Mx2 gene this combination generates a fragment of 996bp. B) Schematic representation of the endogenous Mx2 locus with integrated transactivator and selection cassette. To verify 5´ integration the primer combination c/d was used, resulting in a fragment of 2238bp. For the 3´ integration the primer combination e/f was used generating a fragment of 2258bp. C) PCR fragment analysis of the representative clone ESMx2-tTA ES3-24. PCR was performed using Mx2 and tTA specific oligonucleotides as indicated in A and B. The fragments were analysed on a 1% agarose gel and stained with Medori green.

presumably represent random integration events (Table 22). Furthermore one clone displayed just 5´ integration while the 3’ PCR did not give rise to a fragment of expected size suggesting that integration might have happened in exon 2 without elimination of the endogenous Mx2 gene body. Accordingly, these clones were not further considered.

Theoretically, homologous recombination can occur on one or both of the Mx2 encoding chromosomes and thus might result in a heterozygous or homozygous genotype. Accordingly, after recombination, the endogenous Mx2 gene might be either present in a single copy or deleted (homozygous knock-out). To determine if the tTA gene was targeted to one or both of the cellular Mx2 loci the seven integration positive clones were subsequently analysed for the presence of the endogenous Mx2 gene by PCR. Here oligonucleotides were used binding

in the coding region of the Mx2 gene as indicated by the arrows (Figure 7A). Of all 7 clones positive for 5´ and 3´ integration a fragment of the endogenous Mx2 gene amplified by PCR could be detected (data not shown and Table 22).

Thus, in all of these clones one copy of the transactivator was found to be integrated into the Mx2 locus while the second locus maintained the endogenous Mx2 gene.

From all transfection experiments with the specific gRNAE2 in combination with one of the three specific gRNAE14 correctly targeted cell clones could be obtained.

From 15 clones obtained clones after G418 selection 7 show correct targeting of tTA into one of the endogenous Mx2 alleles (Table 22). This indicates a high efficiency of homologous recombination. It might be worth to mention that in a repetition experiment using the gRNA combinations E2 and E14.3 a total of 42 G418 resistant clones were identified. Further characterization again revealed a specific integration efficiency of transactivator and selection cassette in 50% of clones (data not shown).

Table 22: Analysis of G418 resistant clones obtained from different gRNA combinations

gRNA combination	Clones obtained	5´ + 3´ integration	Only 5´ integration	Only 3´ integration	No specific product (random integration)	Homo-or heterozygous
E2 + E14.1	1	1	0	0	0	hetero
E2 + E14.2	7	1	1	0	5	hetero
E2 + E14.3	7	5	0	0	2	hetero

For the subsequent studies ESMx2-tTA clone 3-24 was used which was obtained from the gRNA combination E2+E14.3. In the following, this clone is assigned ESMx2-tTA.

3.1.3 IFN dependent expression of Mx2 driven tTA is augmented upon differentiation

Based on the genomic analysis in ESMx2-tTA embryonic stem cells one copy of the Mx2 gene was replaced by the synthetic tTA transactivator after recombination. This allowed the direct comparison of transcriptional activity of the targeted tTA transactivator and the endogenous Mx2 gene on the level of mRNA. Therefore, the ESMx2-tTA cells were treated with

500U/ml IFN-β for 24 hours. Afterwards the cells were harvested and total RNA was isolated. Subsequently, RNA was reverse transcribed into cDNA and subjected to qRT-PCR analysis using oligonucleotides specific for the transactivator, Mx2 and β-actin as a reference gene.
In the presence of interferon, an increase of relative transcript abundance was measured in the ES cell state for Mx2 as well as for the tTA transactivator mRNA (Figure 8A). In the case of Mx2 transcripts a 4.3 fold induction after IFN treatment was detected. A similar induction rate was observed for tTA transcripts with a mean fold of 4.8 confirming that both genes are regulated consistently. However, the overall induction rate was unexpectedly low given that IFN is known to be a strong inducer of the Mx2 gene. In previous studies based on mouse NIH3T3 cells, an induction rate of about 20fold was reported on mRNA level (Pulverer et al., 2010). It was speculated that the difference between the induction observed in ESMx2-tTA cells and the previous reports might be due to the cellular system used. Thus, it was asked if the cellular differentiation state accounts for the observed discrepancy. Therefore it was tested if the IFN response is modulated in ESMx2-tTA ES cells upon differentiation.

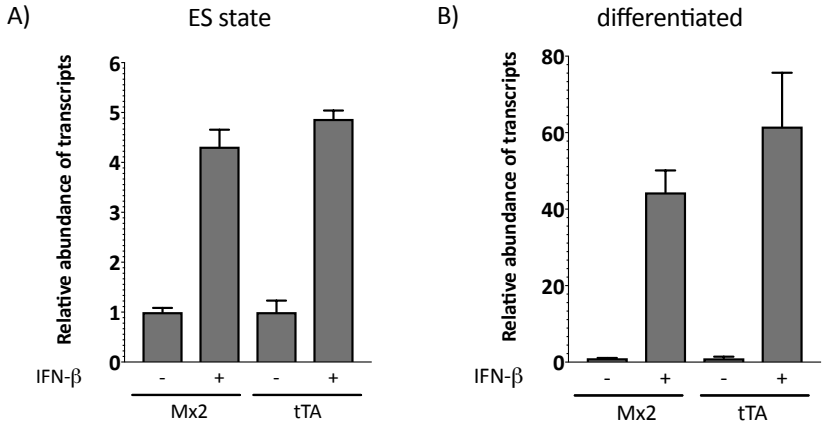

Figure 8: Expression analysis of Mx2 and tTA transcripts after IFN stimulation in the ES cell state and upon differtiation.

To induce differentiation ESMx2-tTA cells were cultivated five days in absence of LIF on gelatine coated 24-well plates. Then, cells were stimulated with 500U/ml IFN -β for 24 hours. Total RNA was isolated and reverse transcribed into cDNA and subsequently analysed by qRT-PCR using sequence specific oligonucleotides for Mx2 or tTA, respectively. The graphs shows the expression level relative to β-actin in the ES cell state (A) and after five days of differentiation (B) related to untreated cells. (n=3).

To differentiate, the ESMx2-tTA cells were seeded on gelatine coated plates and cultivated for five days in the absence of leukaemia inhibitory factor (LIF). This factor is included in the standard media of mES cells and keeps these cells in an undifferentiated state while in absence

of this factor cells differentiate (Murray & Edgar, 2001). Subsequently, the cells were stimulated with 500U/ml IFN-β for 24 hours and gene expression was analysed as described before. Notably, upon differentiation a highly improved expression was detected for Mx2 with a 44fold induction of mRNA levels (Figure 8B). Similar to endogenous Mx2 transcripts also the expression of the tTA transactivator improved upon differentiation. A 62-fold induction was detectable if compared to non-treated cells. Thus, the IFN response in ESMx2-tTA embryonic stem cells is highly attenuated and can be increased upon differentiation.
Moreover, the similar expression properties of tTA and Mx2 upon IFN stimulation both in the stem cell state and upon differentiation confirmed that tTA expression in ESMx2-tTA cells reflects the physiological IFN response. Thus, the targeted integration of tTA into the chromosomal Mx2 locus could transfer the IFN sensitivity to the synthetic transactivator tTA.

3.2 Establishment of IFN and doxycycline sensitive cells

3.2.1 Establishment of IFN dependent reporter cells

The analysis of the targeted embryonic stem cells (ESMx2-tTA) in the previous chapter revealed an authentic IFN dependent induction of the synthetic transactivator RNA. In the next step the activity of the recombinant transcription factor was coupled to reporter gene expression. Therefore, ESMx2-tTA cells were seeded on gelatine and feeder coated 6-well plates and transfected with a circular reporter plasmid containing a transactivator dependent firefly luciferase cassette. Firefly luciferase was chosen as a reporter because of its short half-life of only 3 hours (Thompson et al., 1991) which makes it suitable for monitoring dynamics of gene expression. Furthermore, this reporter can be used to unravel IFN dependent temporal resolution *in vivo* due to its high sensitivity. To select for stable integration into the cellular genome, a hygromycin phosphatase expression cassette under the control of a constitutive promoter was co-transfected. One day after transfection hygromycin B was added and subsequently, a hygromycin B resistant, pool population was obtained assigned as ESMx2-tTA-Luc. This cell pool was then analysed with respect to luciferase expression upon IFN stimulation. ESMx2-tTA-Luc cells were seeded on gelatine coated 24-well plates and stimulated with 500U/ml IFN-β for 24 hours. Cell lysates were generated and luciferase

activity was measured as relative light units (RLU) and related to the total protein amount of the cell lysate.

In the undifferentiated state ESMx2-tTA-Luc cells revealed a twofold reporter induction in response to IFN (Figure 9A). A mean luciferase activity of 1.4X10^{5}RLU/mg was monitored in presence of interferon. Additionally, it was tested if the synthetic circuit could be inhibited by doxycycline which binds tTA transactivator and is expected to impair binding to its cognate promoter driving luciferase expression. In the presence of doxycycline luciferase levels were reduced to 0.4-0.6X10^{5}RLU/mg irrespective of the presence of interferon. This is comparable to luciferase expression in ESMx2-tTA cells in absence of IFN. These values reflect the basal expression of luciferase (tTA independent). After chromosomal integration the expression of a transgene is also influenced by the surrounding host chromatin, irrespective of random or targeted integration. This effect is collectively referred to as "position effects" (Wilson et al., 1990). These effects can lead to tight or leaky expression and determine if the induced transgene expression is sufficient to produce detectable phenotypes (Markstein et al., 2008).

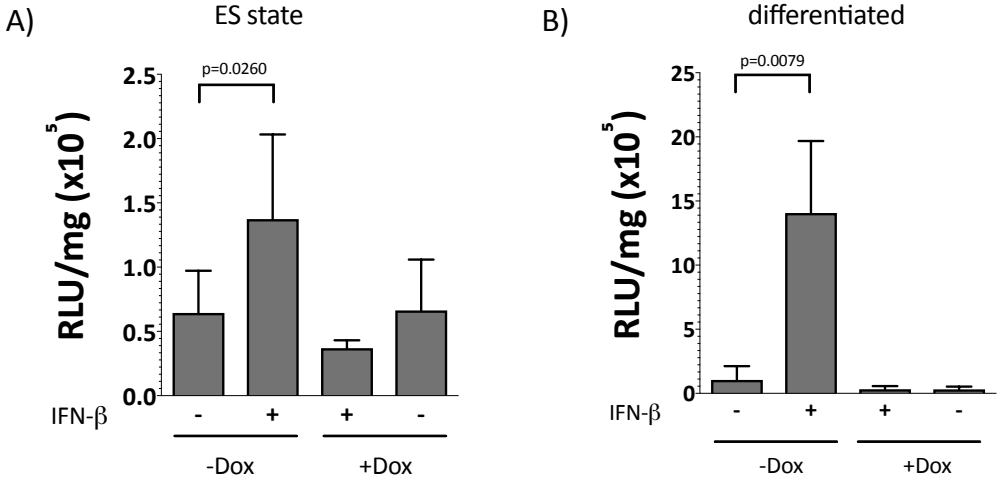

Figure 9: IFN dependent and doxycycline regulatable luciferase expression in undifferentiated ESMx2-tTA-Luc cells and upon differentiation.

ESMx2-tTA-Luc cells were stimulated with 500U/ml IFN-β in presence or absence of 2µg/ml doxycycline (A). For the differentiation, the cells were cultivated for five days in absence of LIF prior stimulation (B). 24 hours after IFN stimulation cells were harvested and luciferase activity was determined and related to the total protein amount as described in the Materials and Methods. (n=3-6).

To test if the tTA dependent luciferase expression could be increased upon differentiation the ESMx2tTA-Luc cells were seeded on gelatine coated 24-well plates and cultivated in the absence of LIF for five days. Afterwards, cells were stimulated with 500U/ml IFN-β for 24 hours (Figure 9B). The luciferase expression was determined as described earlier.

Of note, the differentiation of the ESMx2-tTA-Luc cells improved the functionality of the synthetic circuit dramatically. In the presence of IFN a 13.5 fold induction was detected with mean values of $14X10^5$RLU/mg. In comparison to the ES cell state this reflects a sevenfold induction in presence of IFN in differentiated cells. Noteworthy, the doxycycline dependent repression of reporter expression was still given reducing luciferase expression to basal levels even in the presence of IFN-β.

By comparing the basal luciferase expression rates in absence of IFN no significant difference between the mES state ($0.6X10^5$RLU/mg) and upon differentiation ($1X10^5$RLU/mg) was observed. Thus, the differentiation process had no influence on basal reporter expression. In contrast it improves the responsiveness to IFN dramatically which is in line with the increased mRNA levels that were detected for the tTA (see chapter 3.1.3). This reflects the tight control of the system in the ES state as well in the differentiated state.

3.2.2 Amplification of IFN dependent reporter activity

The ESMx2-tTA-Luc cells displayed a certain degree of sensitivity to IFN as represented by luciferase reporter expression. The overall mean reporter expression in presence of 500U/ml IFN-β was $14X10^5$RLU/mg in differentiated cells. The signal strength is mainly dependent on the concentration of tTA molecules which induce subsequently expression of luciferase by binding to their cognate promoter sequence. In the so far used ESMx2tTA-Luc cells a single tTA gene copy is targeted into the endogenous Mx2 locus. The number of tTA molecules after IFN induction and thereby also the level of the reporter expression is limited by the strength of the endogenous Mx2 promoter. Thus, it was evaluated if the tTA expression level can be increased by the integration of an autoregulatory positive feedback loop to increase the cellular tTA concentration (Figure 10).

To this end, a circuit was implemented in the cells in which the Mx2 promoter controlled tTA additionally activates its own expression, so that the cellular tTA expression levels – and thereby also the levels of the genes controlled by the pTet promoter – would be boosted. Therefore, ESMx2-tTA cells were seeded on gelatine coated 6-well plates and co-transfected with the plasmid encoding for pTet-tTA (harbouring the tTA transactivator under pTet control as well as a constitutive expression cassette for hygromycin phosphoptransferase) as well as a transactivator dependent luciferase reporter plasmids.

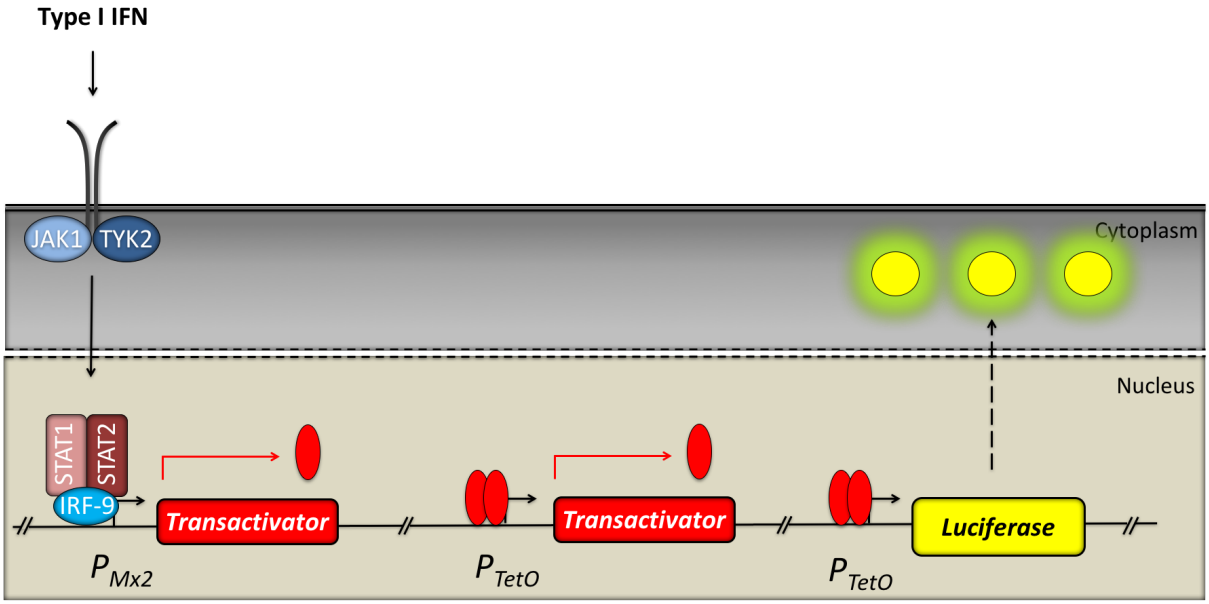

Figure 10: Schematic representation of the autoregulatory circuit.

The IFN dependent expressed transactivator drives expression of transgenes from the tTA dependent promoter P_{TetO}. tTA dependent luciferase expression is amplified by an autoregulatory unit in which the tTA is expressed from the P_{tet} promoter and induces its own expression in a positive feedback.

One day after transfection selection was performed in presence of 300U/ml hygromycin B as described before. After the selection process a pool population of hygromycin B resistant cells was established assigned ESMx2-tTA-Autoluc cells. To test their expression properties these cells were stimulated in the presence of 500U/ml IFN-β for 24 hours. Afterwards cells were harvested and luciferase activity was determined as described before.

In the ES state there was a high degree of luciferase expression detectable already in the basal state with a mean value of $44.9X10^5$ RLU/mg (Figure 11A). Unexpectedly, no further induction was achieved in presence of IFN. However, if the cells were cultivated in the presence of doxycycline luciferase activity was found to be decreased to $0.6X10^5$RLU/mg in IFN stimulated cells and $0.9X10^5$RLU/mg in unstimulated cells. The downregulation in the doxycycline conditions indicated that the luciferase activity in ESMx2tTA-Autoluc cells was strictly tTA transactivator dependent. Notably, the expression in presence of doxycycline was comparable to the expression in ESMx2-tTA-Luc cells which lack the autoregulated loop (Figure 11A).

To evaluate if the IFN dependent regulation is improved in cells that better respond to IFN the ESMx2-tTA-Autoluc cells were differentiated for 5 days as described before and subsequently stimulated with 500U/ml IFN-β for 24 hours. Interestingly, after differentiation of ESMx2-tTA-

Autoluc cells a specific response to IFN was monitored (Figure 11B). Upon IFN stimulation the luciferase activity was increased to 60X10^{5}RLU/mg which corresponds to a 5.5fold induction related to the basal expression. The relative moderate fold induction is due to a relative high basal luciferase expression in absence of interferon which is in the range of basal luciferase expression in the ES cell state (Figure 9A). Also here there is a leaky expression derived from the autoregulated unit. While the cells differentiated they also overcome the attenuation of IFN signalling which makes them sensitive to IFN. In contrast to the ES cell state addition of IFN increases luciferase expression as expected. This suggests that in the stem cell state the autoregulated cassettes are somehow impaired. Interestingly, in the presence of doxycycline the luciferase activity in presence and absence of IFN could be reduced to levels comparable with the ES state and doxycycline presence. This indicates that the high basal expression is tTA dependent.

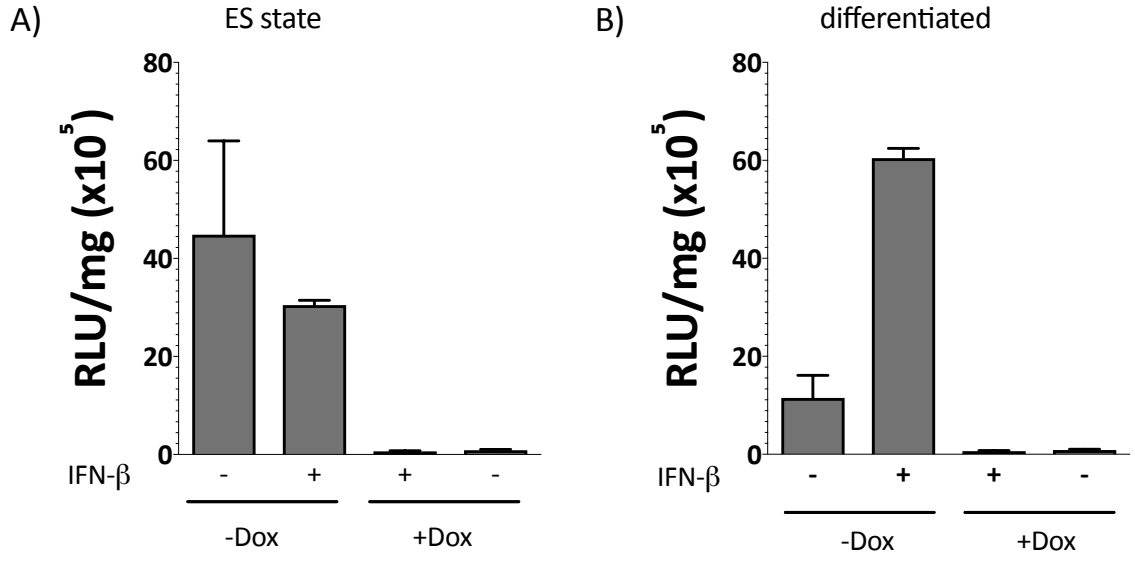

Figure 11: IFN regulation of the amplification circuit is improved upon differentiation.

The ESMx2-tTA-Autoluc cells were seeded and stimulated for 24 hours with 500U/ml IFN-β the next day (A). For differentiation the cells were kept for five days in absence of LIF prior stimulation with 500U/ml IFN-β for 24 hours (B). Luciferase activity of cell lysates was related to total protein content. (n=3).

When comparing the IFN response in ESMx2-tTA-Autoluc cells and ESMx2-tTA-Luc cells it becomes obvious that the implementation of the autoregulatory unit could amplify the IFN response. The ESMx2-tTA-Luc cells displayed a mean luciferase activity of 14X10^{5}RLU/mg at 500U/ml IFN-β whereas the ESMx2-tTA-Autoluc cells amplified this signal to 60X10^{5}RLU/mg.

The luciferase expression induced by the tTA from the Mx2 promoter can be increased through the additional integration of pTet-tTA cassettes thereby increasing luciferase activity.

3.3 Epigenetic modulation of reporter constructs in ES cells over culture time

3.3.1 Loss of luciferase expression upon passaging ESMx2-tTA-Luc cells

There are some reports that indicate that Tet-promoters in embryonic stem cells are negatively affected by epigenetic mechanisms (Kues et al., 2006; Oyer, Chu, Brar, & Turker, 2009, Goedecke at al, 2017). The underlying mechanisms could be attributed to modifications of histones as well as methylation of DNA. Both regulatory processes have a negative impact on reporter expression from pTet-cassettes driving reporter expression (Kues et al., 2006; Oyer et al., 2009). To monitor if IFN dependent luciferase expression is affected in a similar fashion in the ESMx2-tTA-Luc cells the cells were analysed at different time points with respect to their culture maintenance. In particular, cells within passage 3 and passage 11 were first differentiated and then analysed with respect to luciferase expression after IFN stimulation (Figure 12A).

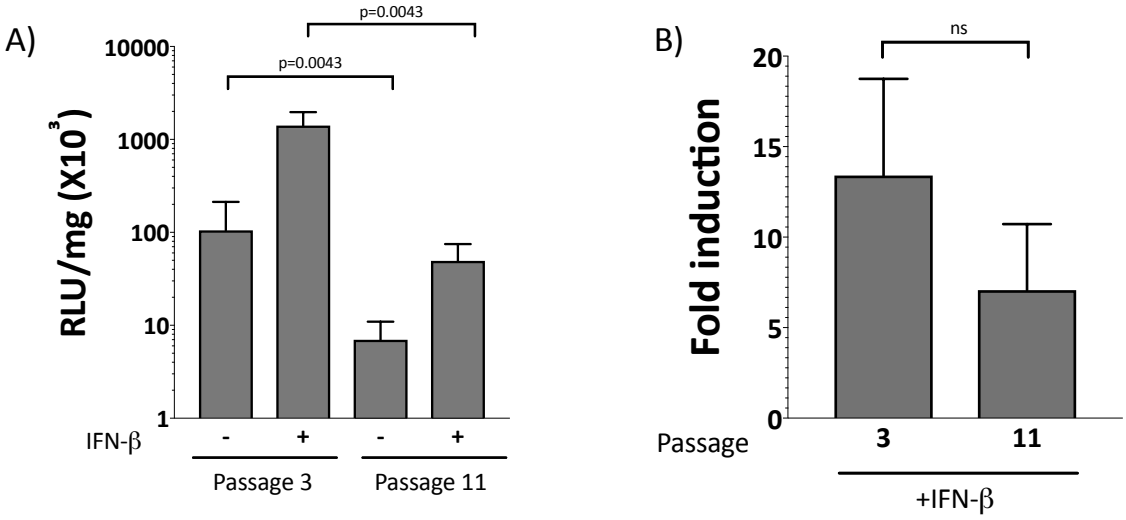

Figure 12: Long-term cultivation of mES cells decreased overall luciferase activity upon differentiation.

Luciferase expression of ESMx2-tTA-Luc cells was analysed after differentiation at different time points of cultivation. Therefore cells of passage 3 and 11 were differentiated for five days in absence of LIF and subsequently stimulated with 500U/ml IFN-β for 24 hours. Cells were harvested and the relative luciferase expression was determined and related to the total protein amount (A). Fold induction rates after IFN stimulation compared to basal expression levels in different passages (B) (n=5-6).

Therefore cells were differentiated in absence of LIF for 5 days and subsequently the cells were stimulated with 500U/ml IFN-β for 24 hours. Afterwards luciferase activity was determined and related to the total protein amount. Of note, a significant decrease in basal luciferase expression was observed from cells differentiated in passage 3 or passage 11. Along with this the cells also responded less to the stimulation with IFN over time.

While cells differentiated from passage 3 showed basal levels of $10X10^4$RLU/mg these levels were decreased in cells differentiated from passage 11 to $0,7X10^4$RLU/mg. Along with this in presence of IFN the activity decreased significantly from $140X10^4$RLU/mg in passage 3 to $5X10^4$ RLU/mg in passage 11. However, the overall fold capacity upon stimulation with IFN did not change significantly between the passages (Figure 12B). Although IFN could induce luciferase expression the overall sensitivity is drastically reduced. This suggests that the overall capacity of reporter induction is not abolished and vice versa that the tTA expression upon IFN treatment is not affected. These results point towards *TetO* affecting silencing mechanisms during cultivation in the mES cells that reduce basal expression but do not abolish tTA based activation.

To evaluate if silencing of the luciferase expression is accompanied with DNA methylation ESMx2-tTA-Luc embryonic stem cells from passage 12 were differentiated in the presence of the DNA methyltransferase inhibitor decitabine. Then, the cells were induced with 500U/ml IFN-β. Treatment with decitabine restored basal luciferase expression 3.7 fold from $0.71X10^4$RLU/mg to $2.6X10^4$RLU/mg (Figure 13A). In the presence of IFN an increase from $4,9X10^4$RLU/mg in absence and $17X10^4$RLU/mg in presence of decitabine corresponded to a similar fold induction of 3,5. Similar to the fold induction rates detected between early and late passages also the treatment with decitabine had no impact on the overall induction rates after IFN stimulation (Figure 13B). This proved that upon extended cultivation luciferase expression is negatively influenced by epigenetic silencing in particular by DNA methylation.

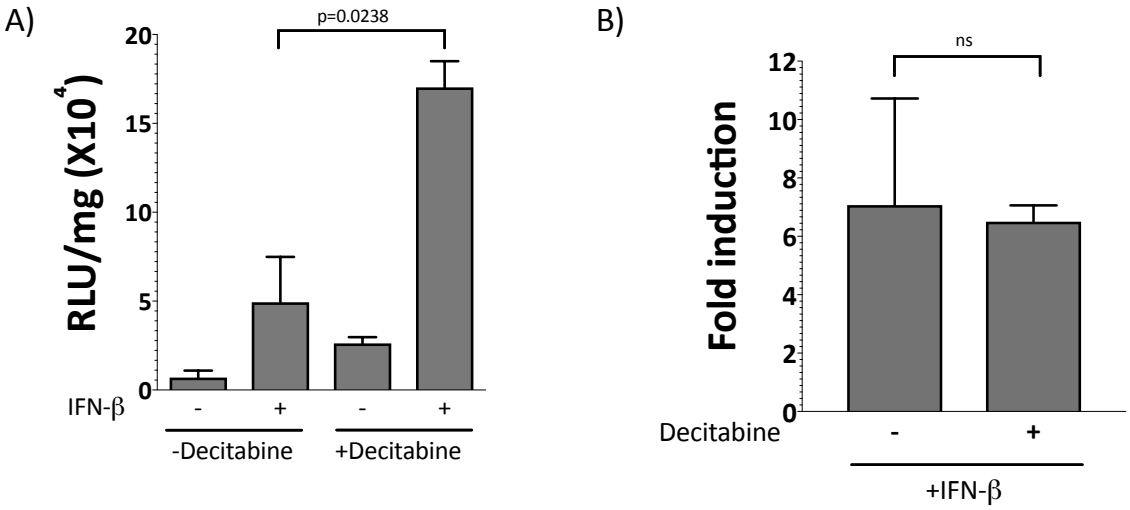

Figure 13: Decitabine partially rescues luciferase activity in ESMx2-tTA-Luc cells upon differentiation.

ESMx2-tTA-Luc cells were differentiated in absence of LIF for 5 days on gelatine coated 24-well plates. Simultaneously 1µM decitabine was included in the culture media. Cells were stimulated with 500U/ml IFN-β for 24hrs. Luciferase activity was analysed and correlated to the total protein amount (A) and the fold induction was determined related to unstimulated cells. (B) (n=3-6).

3.4 Immortalization of differentiated ESMx2-tTA cells and integration of tTA dependent luciferase reporter

3.4.1 Immortalization of ESMx2-tTA cells after differentiation

The limitation in Mx2 promoter activity upon IFN stimulation in ESMx2-tTA-Luc mES cells made the process of differentiation necessary for a functional IFN response. However, *in vitro* differentiated cells have a limited proliferation capacity. Further, long term culture of the ESMx2-tTA-Luc mES cells reduced the capacity to respond to IFN, presumably as a consequence of epigenetic silencing of the luciferase reporter. Together, the so far established cells do not represent a straightforward tool to rewire IFN signalling. Therefore, it was tested if the establishment of a differentiated cell line from ESMx2-tTA cells and subsequent introduction of the luciferase reporter would provide a more robust cell system. To this end, ESMx2-tTA cells were first differentiated and then immortalized to ensure unlimited proliferation.

To achieve immortalisation, the ESMx2-tTA cells were seeded on 6-well plates and cultivated in absence of LIF to induce differentiation. When cells became confluent, they were passaged in a 1:4 ratio. After ten days of cultivation cells were infected with a recombinant lentivirus

transducing SV40 T antigen according to a previously published protocol (Schwerk et al., 2013) (performed by Dr. Tobias May, Inscreenex). Subsequently the cells were selected for their unlimited proliferative capacity over time. Cells which have not been infected would eventually stop proliferation after differentiation whereas cells expressing T-antigen would continue proliferation and expand. After two weeks of continuous culturing an immortalized cell line derived from ESMx2-tTA was established and assigned DIMx2-tTA.

To prove the differentiated state and loss of pluripotency DIMx2-tTA cells were stained for the presence of alkaline phosphatase (AP). AP is an indicator for undifferentiated ES cells (O'Connor et al., 2008). Therefore DIMx2-tTA and G4B12 control ES cells were cultivated for five days at low to medium density in their respective culture conditions. Afterwards cells were fixed with PBS containing 4% paraformaldehyde. Fixed cells were stained with a mixture of Naphthol/Fast red violet (Milipore) to detect alkaline phosphatase activity which stains the cells in red colour. Microscopic analysis for reddish cells was performed (Figure 14).

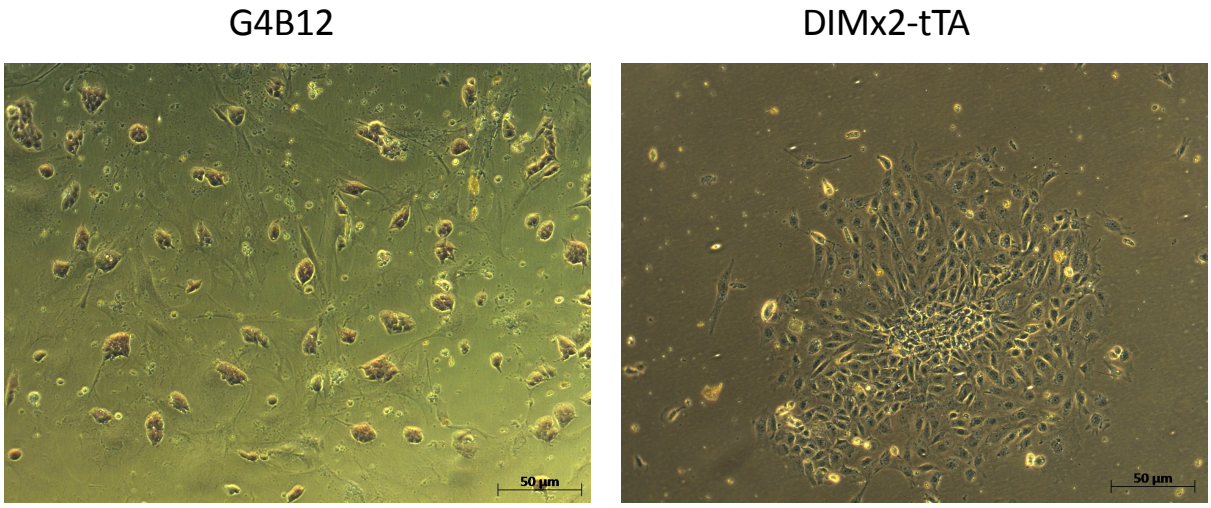

Figure 14: Loss of alkaline phosphatase activity and morphological alterations in DIMx2-tTA cells indicating differentiation.

G4B12 control mES cells and DIMx2-tTA cells stained for presence of alkaline phosphatase to prove the differentiated phenotype of the immortalized cells. Therefore cells were fixed in 4% paraformaldehyde/PBS solution and stained Naphthol AS-BI phosphate. The figure shows representative images of the two cultures.

ES cells like G4B12 appear as a round shaped cell mass and are stained red. In contrast, DIMx2-tTA cells were found to remain unstained indicating lack of alkaline phosphatase activity. Furthermore, these cells displayed many irregular cytoplasmatic extensions. A pale oval nucleus was visible with distinct nucleolus. Additionally the DIMx2-tTA cells were mobile in

contrast to embryonic stem cells. Phenotypically these are indications for a fibroblast-like phenotype. This cell line was used for the subsequent studies.

3.4.2 Functionality of IFN dependent reporter expression in DIMx2-tTA cells

To generate luciferase reporter cells from immortalized DIMx2-tTA cells these were transfected with the transactivator dependent firefly luciferase cassette together with a hygromycin B phosphotransferase selection cassette. Two pool populations of hygromycin resistant cells as well as 13 individual, randomly selected cell clones were isolated. The individual populations were subsequently evaluated for their IFN dependent luciferase activity. Cells were stimulated with 500U/ml IFN-β and luciferase activity was determined 24 hours later. Stimulation with IFN resulted in an induction of the reporter plasmid in some of the established cell populations (Figure 15A). Based on the fold induction (Figure 15B) the pool population 1 (P1) and clone 10 (designated C10) and clone 13 (designated C13) were selected for further investigations.

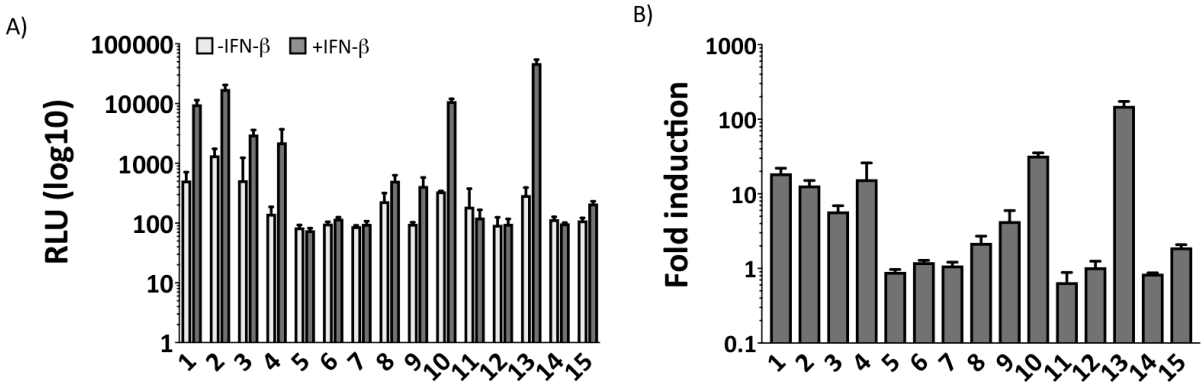

Figure 15: Screening of DIMx2-tTA-Luc IFN dependent reporter cells.

DIMx2-tTA cells were transfected with a transactivator dependent luciferase plasmid (pRBT1Luc) and selected for resistance towards hygromycin B. Both, pool populations (1 and 2) as well as several clones (3-15) were isolated. These populations were stimulated with 500U/ml IFN-β and luciferase activity was analysed as described before. Relative light units (A) and fold inductions compared to control were determined (B) (n=3).

To test if the transactivator dependent functionality is preserved in the DIMx2-tTA-Luc cells were stimulated with 500U/ml IFN-β in presence or absence of 2µg/ml doxycycline. After 24 hours cell lysates were harvested and luciferase activity was determined and related to total protein amount.

With regard to the overall activity, the DIMX2-tTA-Luc reporter cell lines P1 and C10 displayed similar induction levels (Figure 16A, B). However, the highest activity of luciferase in presence

of IFN was given in C13 with a mean RLU/mg of 48X10^{5}RLU/mg (Figure 16C). Based on these values, the fold induction was calculated to be 42 for P1, 32 for C10 and 48 for C13. Thus, the immortalized DIMx2-tTA-Luc cells maintained the responsiveness to IFN and furthermore the overall sensitivity was highly improved compared to ESMx2-tTA-Luc.

The IFN dependent luciferase expression could still be downregulated in presence of doxycycline (Figure 16). Doxycycline treatment reduced transactivator dependent luciferase expression to basal levels even in presence of IFN. This proves the lack of basal activity of transactivator or luciferase in absence of IFN.

Together, the established differentiated and immortalized cells DIMx2-tTA-Luc displayed a highly improved sensitivity to IFN in comparison to the ESMx2-tTA-Luc. The functionality of IFN dependent tTA expression and subsequent transmission to luciferase reporter cassettes could be preserved. Thus, the DIMx2-tTA-Luc cells represent a robust system for subsequent analysis.

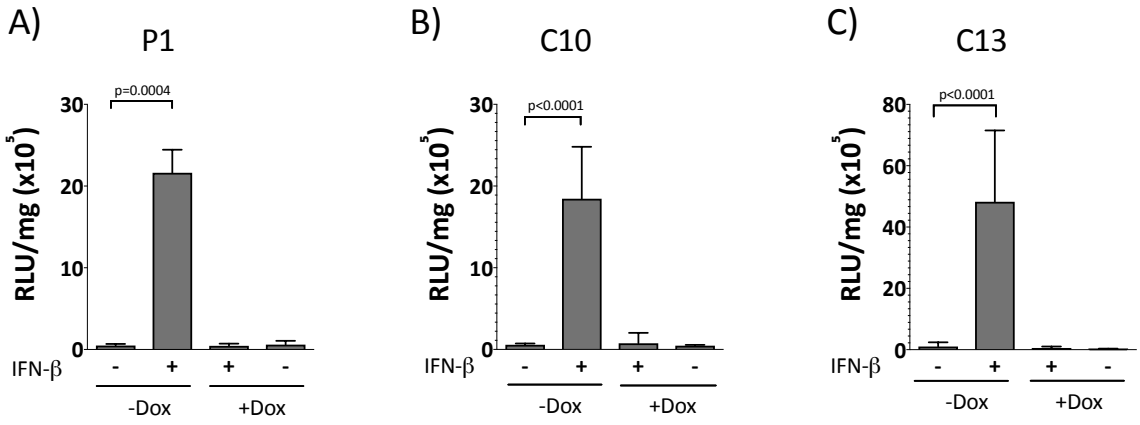

Figure 16: Doxycycline dependent tTA regulation in DIMx2-tTA-Luc reporter cells.

The pool population (P1) as well as two distinct clones (C10 and C13) were stimulated with 500U/ml IFN-β in presence or absence of 2µg/ml doxycycline. After 24 hours cells were harvested and the luciferase activity was determined and related to the total protein amount (n=6-11).

3.4.3 Temporal resolution of IFN dependent reporter expression

The DIMx2-tTA-Luc reporter cells rewire IFN signalling to a quantifiable luciferase reporter. In the first place IFN induces the expression of the tTA transactivator which in turn binds to its cognate DNA sequences and induces expression of luciferase. In the end two transcriptional and translational steps are needed after IFN recognition to detect reporter expression. To

monitor the temporal resolution of the circuit DIMx2-tTA-Luc P1 and C10 were stimulated with 500U/ml IFN-β. Cells were harvested 1,5-24 hours later and luciferase activity was analysed (Figure 17). Both cell populations displayed a similar kinetic of IFN dependent luciferase activity. Already at 1.5 hours post stimulation a statistically significant increase was detectable. For DIMx2-tTA-Luc P1 a 4-fold induction and for DIMx2-tTA-Luc C10 a 3-fold induction was measured. After 3 hours the response in both populations highly increased and reached a maximum at 6 hours.

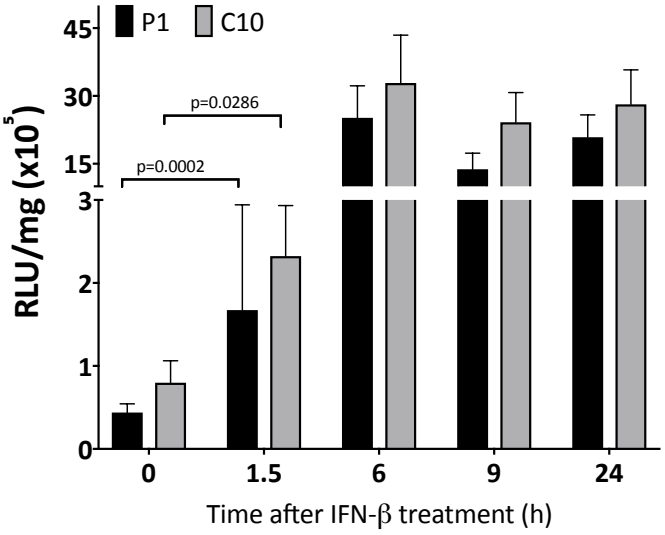

Figure 17: Temporal resolution of IFN dependent luciferase activity.

DIMx2-tTA-Luc P1 and C10 cells were stimulated with 500U/ml IFN-β and harvested at indicated time points. Relative luciferase activity is presented. DIMx2-tTA-Luc P1 is displayed as black bars and DIMx2-tTA-Luc C10 is displayed as red bars (n=4-10).

3.4.4 IFN dependent reporter expression in DIMx.2-tTA-Luc cells

Since all DIMx2-tTA-Luc IFN dependent reporter cells (P1, C10 and C13) were originally derived from the parental ESMx2-tTA cell line the gene copy number of the tTA transactivator is one as a consequence of targeted integration into one of the two endogenous alleles (see chapter 3.1.3). This suggests that upon stimulation with a specific concentration of IFN the amount of transactivator molecules within the cell is similar in all the DIMx2-tTA populations. However, the transactivator dependent luciferase reporter was introduced randomly. Thus, the integration sites as well as the copy numbers of the reporter plasmids are not known for the

three DIMx2-tTA-Luc populations P1, C10 and C13. This raised the question if the mode of reporter cassette integration has an influence on the sensitivity to IFN in the different cell populations. In order to analyse this the DIMx2-tTA-Luc populations P1, C10 and C13 were stimulated with different concentrations of IFN-β and luciferase activity was determined after 24 hours. All three DIMx2tTA-Luc populations displayed a significant increase in reporter expression at concentrations as low as 5U IFN-β. This resulted in a fold induction of 3 to 4 compared to non-treated cells for P1 and C10. All tested cell populations displayed a highly increased luciferase activity at concentrations of 50U IFN-β. Interestingly, stimulation with different concentrations of IFN-β resulted in comparable levels of luciferase in DIMx2-tTA-Luc cells P1 and C10 (Figure 18). In contrast, it might be noteworthy to mention that DIMx2-tTA-Luc C13 displayed an increased sensitivity to different IFN concentrations. While the tTA transactivator copy number in all established DIMx2-tTA-Luc populations is one these results indicate that the higher sensitivity may be attributed to a higher copy number of random integrated luciferase cassettes or that they are integrated into more active chromosomal sites.

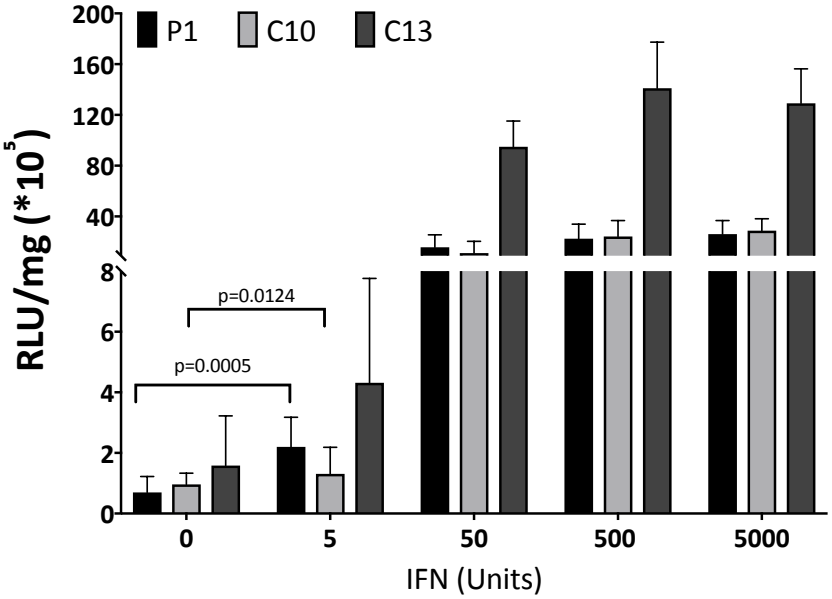

Figure 18: Dose dependent luciferase activity in different DIMx2-tTA-Luc reporter cell populations.

The DIMx2-tTA-Luc P1, C10 and C13 cells were stimulated with indicated concentrations of IFN-β and luciferase activity was determined 24 hours later and related to the total protein amount as described before (n=6-9).

3.4.5 Doxycycline regulatable luciferase expression in presence of IFN in DIMx2-tTA-Luc cells

Since doxycycline can block IFN induced activation of luciferase it was investigated if doxycycline can be used to fine tune reporter gene expression. To test this DIMx2-tTA-Luc P1 cells were seeded in a 24-well plate format and stimulated the next day with 500U/ml IFN-β to induce transactivator dependent luciferase expression. Simultaneously, different concentrations of doxycycline were added to repress transactivator binding to the pTet promoter. Luciferase activity was determined and related to total protein amount 24 hours later as described before.

The expression of luciferase could be significantly inhibited down to concentrations of 10^{-3}µg/ml doxycycline (Figure 19). At a concentration of 10^{-4}µg/ml approximately 50% of the luciferase activity was obtained. Lower concentrations of doxycycline could not repress luciferase expression. Based on these results the transactivator dependent luciferase expression in presence of IFN can be fine-tuned within a narrow concentration range of doxycycline.

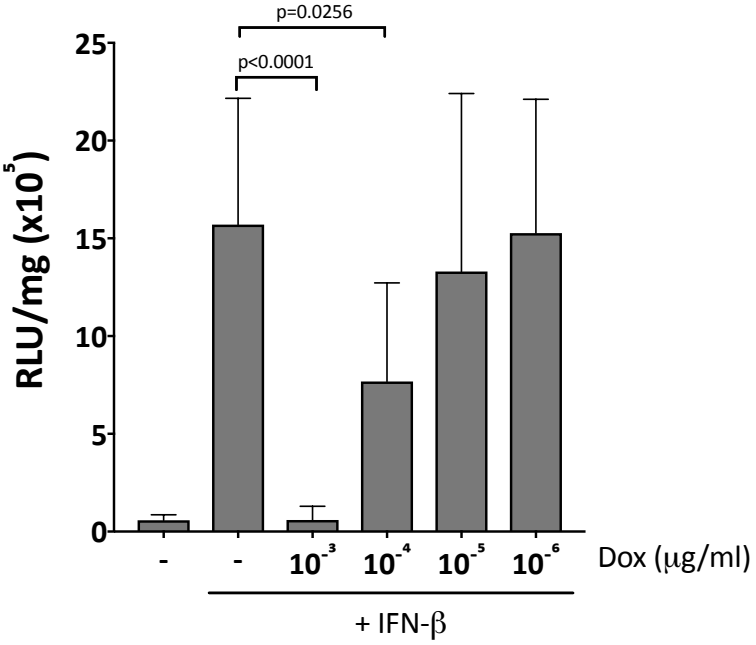

Figure 19: Doxycycline dependent repression of IFN dependent luciferase expression.

DIMx2-tTA-Luc P1 cells were stimulated with 500U/ml IFN-β in presence of indicated concentrations of doxycycline. After 24 hours cells were harvested and the activity of luciferase was determined and related to the total protein amount as described before (n=3-9).

3.5 Rewiring IFN signaling to synthetic cassettes in cells based on BAC vectors

In the so far discussed ESMx2-tTA-Luc and DIMx2-tTA-Luc cells a single gene copy of the tTA transactivator is integrated into the Mx2 locus driving reporter expression in IFN stimulated conditions. It was asked if an increase in tTA transactivator copies would have an influence on the sensitivity to IFN. However, targeting the endogenous Mx locus is limited to a maximum of two copies. To increase the overall copy number of IFN dependent tTA transactivator and to preserve all the necessary regulatory elements of the endogenous Mx2 a different approach was used. To this end, bacterial artificial chromosome (BAC) vectors were selected. BAC vectors have been shown to accurately reflect expression of endogenous chromosomal genes after transfection (Heintz, 2001). They can harbour up to 300kb of genomic sequence including the remote regulatory regions and thus provide all the relevant regulatory sequences.

The BAC RP24-71I6 was used for *in vitro* recombination (Datsenko and Wanner, 2000). This BAC vector comprises the Mx2 locus including 5´ and 3´ sequences of 103kb and 75kb, respectively. It has been shown to reflect a precise regulation and correct spatial and temporal expression of targeted transgenes with respect to IFN stimulation (Pulverer et al., 2010). To generate the recombinant BAC vector, the open reading frame of the murine Mx2 gene was replaced by the tTA gene according to the scheme shown in Figure 20.

For BAC mutagenesis *in vitro* recombination was performed. The linear repair template comprising the tTA transactivator and the selection cassette as well as homology arms was isolated from pUHD15-1-Mx2HR-tTA after enzymatic restriction. The 3.7kb fragment was electroporated in the bacteria expressing the recombination enzymes and containing the BAC-RP27-71I6. Selection was performed in presence of chloramphenicol and kanamycin to select for bacteria carrying the BAC and the transduced repair template, respectively. Chloramphenicol/kanamycin resistant bacterial clones were analysed for successful recombination via colony PCR, thereby employing the oligonucleotides indicated in Figure 7 (data not shown). By this procedure, bacterial colonies were identified that harbour the BAC-Mx2-tTA, in which the tTA was correctly integrated into the desired site. This BAC was used for further purposes.

To establish recombinant cells the linear BAC-Mx2-tTA was transfected into NIH3T3 fibroblasts via lipofection and selected for G418 resistance. Individual clones were obtained and subsequently analysed.

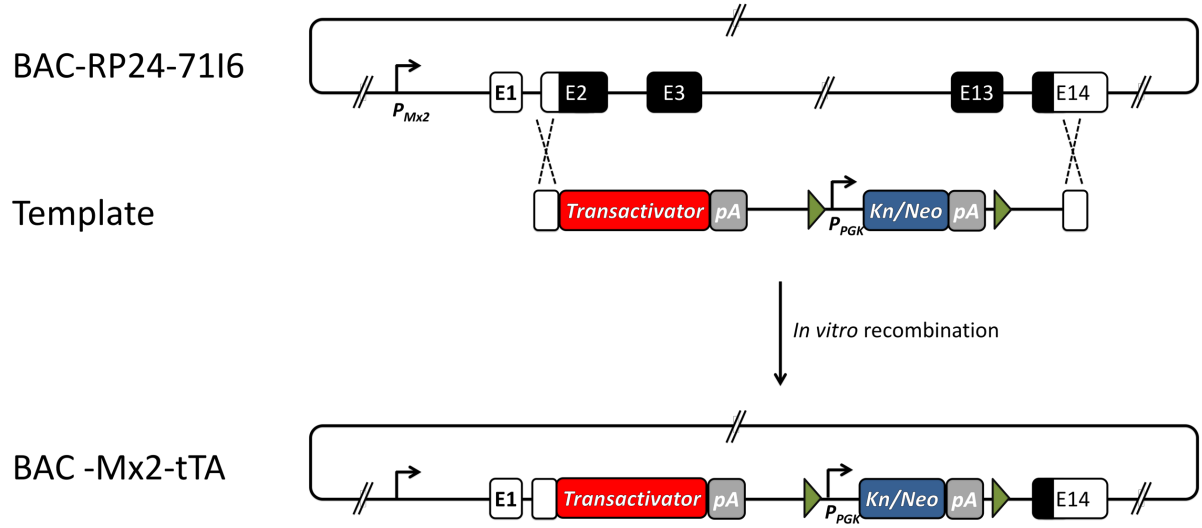

Figure 20: Recombineering strategy for BAC-Mx2-tTA.

The 3.7kb repair template was *in vitro* recombined with the BAC-RP24-71I6 to generate BAC-Mx2-tTA. Therefore the repair template was electroporated into DH10B *E. coli* cells which carry the BAC-RP24-71I6. Selection was performed in presence of kanamycin (template) and chloramphenicol (BAC) to get access to clones where recombination was successful. The homology parts of the repair template homologous to Mx2 are indicated as white rectangles. FRT sites are indicated as green rectangles.

3.5.1 Screening for regulatable BAC Mx2tTA clones

BAC-Mx2-tTA stable transfected NIH3T3 clones were obtained and tested for the highest tTA transactivator activity. Therefore different clones were transient transfected with a tTA dependent luciferase construct in presence or absence of 2µg/ml doxycycline and stimulated the next day with 500U/ml IFN thereby maintaining doxycycline conditions. After 24 hours cells were harvested and luciferase expression was determined and related to the total protein amount. The use of liposomal transfection reagents has been reported to induce IFN synthesis and expression of ISGs (Li et al., 1998). This transient effect of liposomal mediated IFN production was used to screen for doxycycline regulatable clones – a hallmark of the tTA transactivator. As expected, the liposomal transfection of a tTA dependent luciferase construct induced IFN expression and subsequent ISG induction leading to luciferase expression in absence of doxycycline. This is exemplified by clone 2 (Figure 21A). The addition

of IFN had no impact on luciferase expression indicating that the IFN levels reached with lipofection are already saturating. Presence of doxycycline reduced reporter in all tested clones, albeit to different extents. On the basis of this observation the transactivator activity was determined to identify regulatable clones (Figure 21B). The value was reflected by the ratio between absence and presence of doxycycline in IFN stimulated cells. Based on this value the highest transactivator activity could be attributed to clone 2 which represented a fold induction of 20. All the other clones showed lower induction rates of 2-8 fold. Of note, in control NIH3T3 cells that served as an internal control, no difference of luciferase expression was observed in presence or absence of doxycycline.

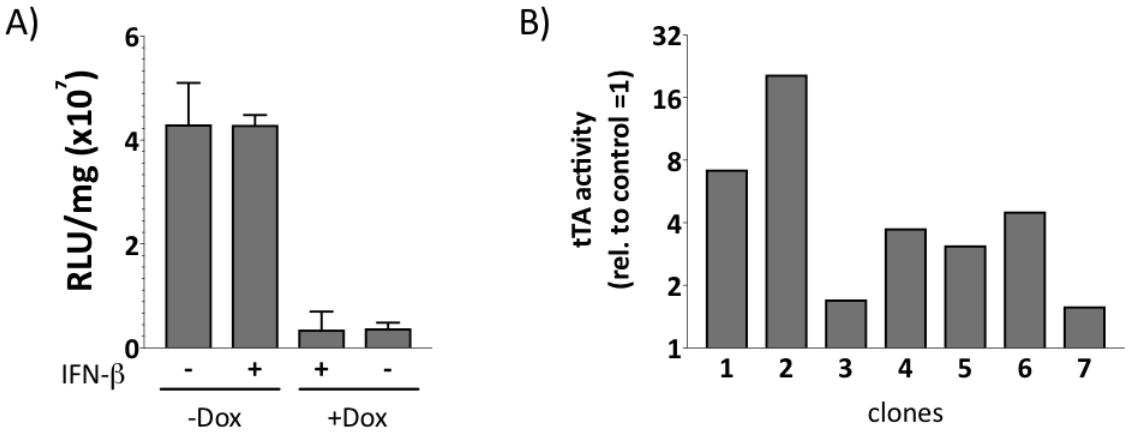

Figure 21: Screening for regulatable NIH3T3 BAC-Mx2-tTA clones.

NIH3T3 fibroblasts were transfected with the BAC-Mx2-tTA and selected for resistance to neomycin as described before. The obtained clones were transiently transfected with a transactivator dependent luciferase reporter (pRBT1luc) in presence or absence of 2µg/ml doxycycline. The next day cells were stimulated with 500U/ml IFN-β along with doxycycline treatment. After 24 hours cells were harvested and luciferase activity was determined and related to total protein amount. BAC-Mx2-tTA clone 2 is presented as a representative clone (A). The activity of the transactivator was determined for seven individual cell clones. This was calculated from the ratio between absence and presence of 2µg/ml doxycycline in presence of 500U/ml IFN-β (B). Control NIH3T3 (ctr) cells were transfected with pRBT1luc and stimulated accordingly (n=3).

Based on the screening approach BAC-Mx2-tTA clone 2 was identified as the clone showing the best tTA transactivator activity. This clone was used for further studies.

3.5.2 Increased sensitivity to IFN in BAC-Mx2-tTA-Luc reporter cells

BAC-Mx2-tTA clone 2 displayed the best tTA transactivator activity in the transient luciferase reporter screening and was used to generate a stable IFN dependent reporter cell line. The

BAC-Mx2-tTA clone 2 was co-transfected with a tTA transactivator dependent luciferase cassette together with a hygromycin B phosphotransferase cassette. A pool population was established designated as BAC-Mx2-tTA-Luc cells. To test IFN dependent luciferase expression BAC-Mx2-tTA-Luc cells were stimulated with 500U/ml IFN-β in presence or absence of 2µg/ml doxycycline. After 24 hours cell lysates were generated and luciferase activity was determined and related to total protein amount.

IFN stimulation of these cells resulted in a 3.8 fold induction of luciferase activity up to $1.8X10^8$ RLU/mg (Figure 22). Of note, a relatively high basal activity of $4.9X10^7$ RLU/mg was detected in BAC-Mx2-tTA-Luc cells in absence of IFN. Interestingly, this basal activity was partly dependent on the tTA transactivator since in presence of doxycycline the basal level could be decreased to $1.7X10^7$RLU/mg. This indicated that there is a certain degree of IFN independent expression of tTA in these cells. This might be explained by partial fragmentation of certain BACs during the random integration process. Thereby regulatory elements necessary for regulation might have got lost resulting in this elevated tTA dependent basal expression.

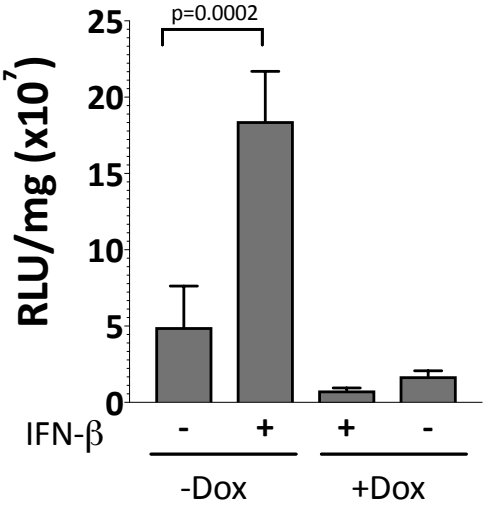

Figure 22: IFN regulation in BAC-Mx2-tTA-Luc cells.

BAC-Mx2-tTA clone 2 cells were stable transfected with pRBT1luc and pHt and selected for hygromycin B resistance. The established pool population was designated BAC-Mx2-tTA-Luc. To test IFN dependent luciferase activity cells were stimulated with 500U/ml IFN-β in presence or absence of 2µg/ml doxycycline. After 24 hours cells were harvested and luciferase activity was determined and related to total protein amount. (n=8).

Comparing the BAC-Mx2-tTA-Luc system with the DIMx2-tTA-Luc C13 cells a 10 fold higher expression level of luciferase could be obtained in presence of IFN. On the other side, the DIMx2-tTA-Luc C13 cells display a clearly better induction rate after stimulation. Whereas the

BAC-Mx2-tTA-Luc cells increase luciferase expression 3.8 fold when IFN was present the DIMx2-tTA-Luc C13 cells displayed a more pronounced (94 fold) induction (values obtained from dose dependency, Figure 18). The minor fold induction observed in the BAC-Mx2-tTA-Luc cells is accounted to the high basal tTA dependent expression. This was totally absent in the DIMx2-tTA-Luc C13 cells. Thus, DIMx2-tTA-Luc C13 cells reflect a much tighter regulation.

3.6 Conversion of IFN to anti-inflammatory signals

3.6.1 IFN dependent expression of mIL10 *in vitro*

Although type I IFN have been assigned to viral and bacterial infections more and more evidence supports its role also in inflammatory processes. Especially in autoimmune syndromes like systemic lupus erythematosus (SLE) a pro-inflammatory role for type I IFNs has been assigned (Baechler et al., 2003). Thus, it was tested if the IFN induced tTA activation could be rewired to an anti-inflammatory response. This would offer the possibility to reduce pathophysiological inflammation induced by IFN.

Due to its multiple, pleiotropic effects with regard to immunomodulation and inflammation IL10 was selected as a model anti-inflammatory cytokine. In order to connect IL10 secretion to transactivator activity a pTet-mIL10 cassette was stably integrated into the DIMx2-tTA and BAC-Mx2-tTA cells. Therefore, cells were transfected with a pTet controlled mIL10 cassette which additionally carried a hygromcycin B selection marker. Cells were selected in the presence of 300U/ml hygromycin B. A pool population was established from both cellular systems designated DIMx2-tTA-mIL10 and BAC-Mx2-tTA-mIL10. For quantitative analysis of mIL10 expression levels upon type I IFN stimulation cells were treated with 500U/ml IFN-β. After 24 hours total RNA was isolated and cDNA synthesis was performed. Afterwards qRT-PCR was performed using mIL10 specific oligonucleotides. As reference gene β-actin was used. qRT-PCR analysis revealed that both cell systems displayed IFN dependent induction of mIL10 transcripts upon IFN stimulation (Figure 23A). Whereas the overall fold induction was similar in both cell system (Figure 23B) the relative expression differed. DIMx2-tTA-mIL10 displayed low basal levels of $2.8X10^{-4}$ and a relative expression of $15X10^{-4}$ in presence of IFN-β. In contrast, BAC-Mx2-tTA-mIL10 cells expressed $10X10^{-4}$ in the basal and $79X10^{-4}$ in the

induced state. In summary, mIL10 could be induced by IFN in both cell systems. While the DIMx2-tTA-mIL10 cells showed less basal levels, the BAC-Mx2-tTA-mIL-10 cells achieved highest levels of mIL10.

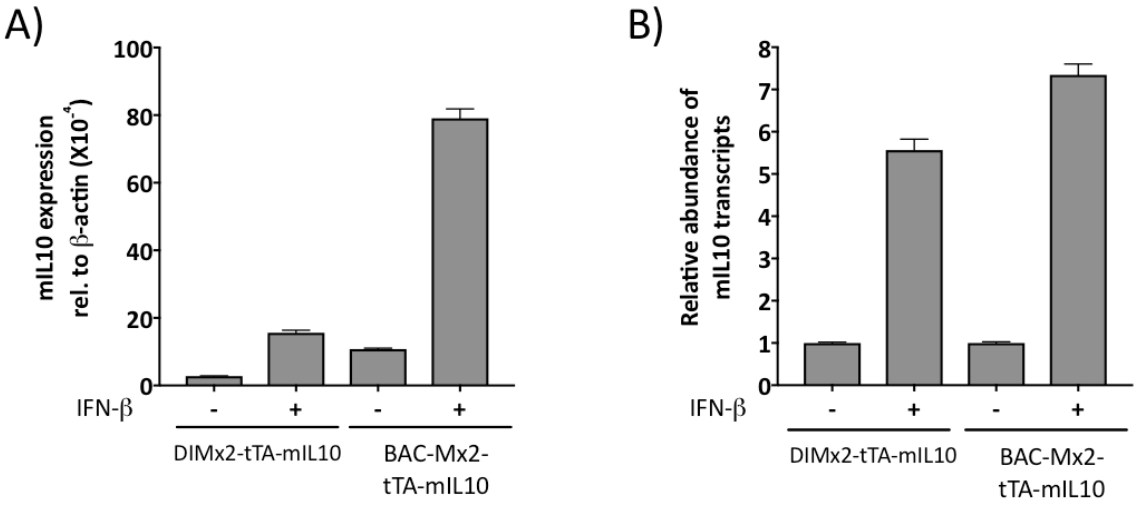

Figure 8: Analysis of IFN induced mIL10 mRNA expression in Mx2-tTA.mIL10 cells.

DIMx2-tTA-mIL10 and NIH3T3-BAC-Mx2-tTA-mIL10 cells were stimulated with 500U/ml IFN-β. After 24 hours cells were harvested and total RNA was isolated. Relative expression of mIL10 mRNA was analysed via qRT-PCR using mIL10 specific oligonucleotides. (A). To determine the relative fold induction rates of both cellular system IFN stimulated cells were related to non-stimulated cells (B) (n=3).

3.6.2 Detection of mIL10 in cell supernatants of BAC-Mx2-tTA-mIL10 after IFN stimulation

The analysis on the mRNA levels displayed that mIL-10 could be induced upon IFN stimulation. To test if the achieved RNA levels were sufficient to translate into detectable mIL10 protein levels the amount of secreted mIL10 was determined after IFN stimulation. Therefore BAC-Mx2-tTA-mIL10 cells were used because they displayed an overall higher relative expression on the transcriptional level. Cells were seeded in a 24-well plate format and stimulated the next day with 500U/ml IFN-β in presence or absence of 2µg/ml doxycycline. After 48 hours the supernatant was collected. Samples were centrifuge at 13.000rpm for five minutes to clarify the supernatant. The mouse IL10 ELISA MAX^{TM} from Biolegend was used according to the user´s manual was used to determine the amount of mIL-10 (Figure 24).

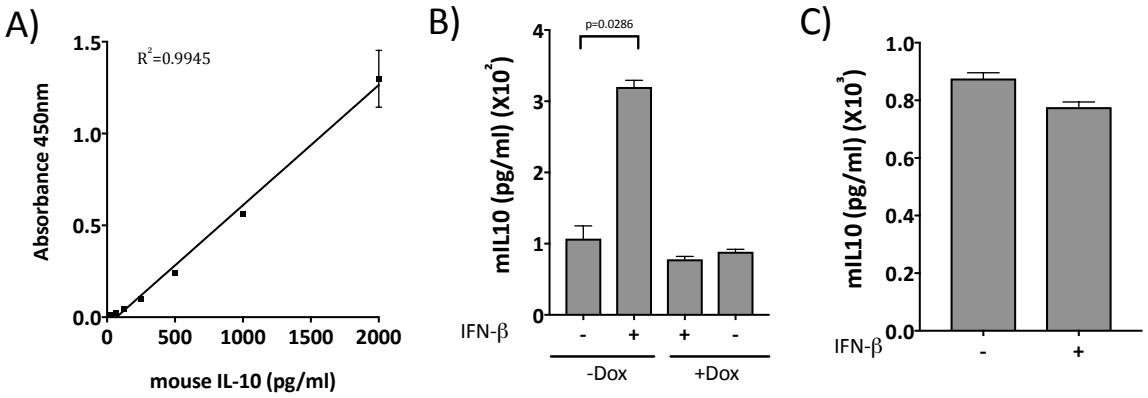

Figure 9: Determination of mIL10 levels in culture supernatants from NIH3T3 BAC-Mx2-tTA-mIL10 cells after IFN stimulation.

mIL10 in cell culture supernatant was detected by ELISA. Reference curve for mIL10 performed with ELISA and used for quantification of IL10 concentrations in cell culture supernatants. R^2=0.9945 (A). BAC Mx2-tTA-mIL10 cells were seeded in a 24-well plate format and stimulated the next day with 500U/ml IFN-β along with 2µg/ml doxycycline. After 48 hours supernatant was harvested and analysed for mIL10 presence via ELISA assay as described within the text (B). Similar NIH3T3 WT cells were treated and analysed (C) (n=4).

The analysis revealed that upon IFN treatment there was an increase of mIL10 in the supernatant with a mean concentration of 320pg/ml (Figure 24B). Compared to non-stimulated conditions IFN enhanced the secretion of mIL10 3 fold. In the presence of doxycycline the levels of IL10 could be repressed to non-stimulated condition. This reflects the tight regulation of the circuit in agreement to what was observed using the luciferase reporter. Moreover, the doxycycline sensitivity indicated that the signal obtained from IL10 is transactivator dependent and not mediated by IFN stimulating endogenous IL10 production. This was also proven by control cells where IFN did not induce endogenous IL10 expression (Figure 24C).

The transmission of pro-inflammatory signalling to induce anti-inflammatory cytokine IL10 secretion proved the modularity of the circuit with regard to transmitting an pro-inflammatory input signal to an anti-inflammatory output. Additionally, this offers the possibility to study the counteracting properties in an infection model.

3.7 Functional evaluation of BAC-Mx2-tTA-Luc and DIMx2-tTA-Luc cells after transplantation into mice

3.7.1 IFN dependent reporter expression upon pathophysiological conditions

The two Mx2-tTA based reporter systems developed in this thesis (DIMx2-tTA-Luc/BAC-Mx2-tTA-Luc) displayed a different sensitivity towards IFN *in vitro*. The DIMx2-tTA-Luc generated reporter cells showed IFN induced luciferase activity in the range of 10^6 RLU/mg. In contrast the BAC-Mx2-tTA-Luc cell displayed a higher sensitivity to IFN with values in the range of 10^8 RLU/mg. To compare their sensitivity upon pathophysiological IFN concentrations the most sensitive cell populations DIMx2-tTA-Luc C13 and BAC-Mx2-tTA-Luc cells were comparatively evaluated *in vivo* upon engraftment into mice. To this end, the two cellular IFN reporters systems were subcutaneously transplanted into immunocompromised *RAG2/IL2rgc* double knockout mice ($1X10^6$cells/transplantation). This mouse strain lacks T cells, B cells and natural killer (NK) cells. This strain is suitable for transplanting allogeneic or xenogeneic cells which are often rejected by NK cells. By this, this model allows the transplantation of cells *in vivo* and can be used for sequential challenges (data not shown). The immunostimulant polyinosinic:polycytidylic acid (polyI:C) was used to mimic an infection in these mice by intraperitoneal injection. PolyI:C stimulates mainly plasmocytoid dendritic cells and macrophages to produce IFN. Due to the microvascularization IFN is systemically distributed within the body of the animal including the grafts. In contrast to the intraperitoneal application of recombinant IFN, a simulation of an infection is used to compare the response to a given physiological endogenous IFN signal. Two engraftments of each cell system were performed per mouse. The DIMx2-tTA-Luc C13 cells were transplanted on the left side of the back whereas the BAC-Mx2-tTA-Luc cells on the right side of the back. Cell transplantation, polyI:C stimulation and *in vivo* imaging was performed as specified in Material and Methods.
The basal expression of luciferase before polyI:C stimulation (24hrs after transplantation) differed already between DIMx2-tTA-Luc C13 ($0.16X10^4$ p/s/cm^2/sr) and BAC-Mx2-tTA-Luc ($5.76X10^4$ p/s/cm^2/sr). Similar to the *in vitro* observation the BAC-Mx2-tTA-Luc cells displayed a general higher basal activity of luciferase. Three hours after polyI:C stimulation response from both grafts were visible: DIMx2-tTA-Luc C13 grafts displayed a 5.7 fold induction compared to basal levels before treatment and kept the signal at a similar level until 24 hours (Figure 25A, C). Also the BAC-Mx2-tTA-Luc cells showed a

significant reporter induction after 3 hours with a 2.9 induction fold. Here the signal declined after 24 hours to a 1.5 fold induction (Figure 25B, D).

Thus, both DIMx2-tTA-Luc C13 and BAC-Mx2-tTA-Luc cell populations showed an induction of luciferase expression after 3 hours which was still measurable after 24 hours. With regard to the temporal resolution there was not a significant difference between both cell systems at the time point investigated.

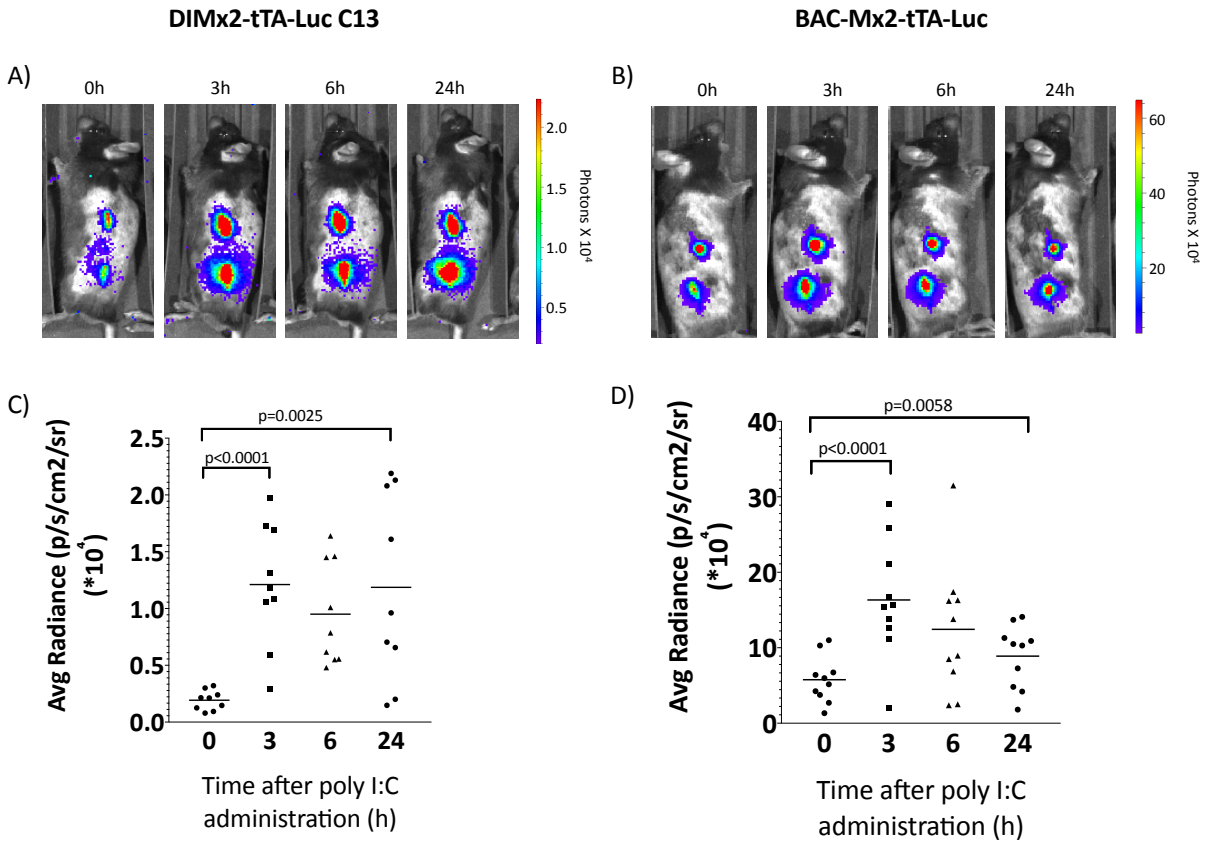

Figure 25: Transmission of IFN signalling to luciferase reporter via DIMx2-tTA-Luc C13 and BAC-Mx2-tTA-Luc.

One million cells were subcutaneous transplanted in *RAG2/IL2rg* mice. Each animal received two cell transplants at the left side of the back and one on the right side of the back. Two days after transplantation the animals were challenged with 200µg poly I:C via intraperitoneal injection. Animals were subjected to bioluminescence imaging at different time points after treatment as described before. (A: DIMx2-tTA-Luc C13 and B: BAC-Mx2-tTA-Luc). Quantification of the bioluminescence signal over the time was performed with "Living Image" software. Each point represents one cell transplantation (C: DIMx2-tTA-Luc C13 and D: BAC-Mx2-tTA-Luc) (n=9). Note that the scale in A and B is different.

However, when comparing the overall luciferase activity in presence of polyI:C induced IFN at three hours post stimulation revealed a 20 fold higher luminescence in BAC-Mx2-tTA-Luc grafts. On the other hand the BAC-Mx2-tTA-Luc grafts displayed a pronounced basal activity and were not as responsive to polyI:C treatment as the DIMx2-tTA-Luc cells. This was reflected

by the lower fold induction at three hours post stimulation. In summary, whereas the BAC-Mx2-tTA-Luc cells reflected a higher sensitivity to polyI:C stimulation the DIMx2-tTA-Luc system showed a better regulation due to its lower basal activity.

3.7.2 Doxycycline dependent regulation of IFN transmitted reporter expression i*n vivo*

Both Mx2-tTA systems used to transmit IFN activity to a luciferase reporter (BAC-Mx2-tTA-Luc and DIMx2-tTA-Luc cells) proved their functionality with regard to reporter expression after polyI:C stimulation. To evaluate if the IFN induced activation of the system can be controlled by doxycycline also *in vivo*, $1X10^6$ DIMx2-tTA-Luc C13 cells/transplant were subcutaneously transplanted into eight *RAG2/IL2-rg* double knockout mice as described before. Each mouse received three cell implants in total, two on the left side of the back and one on the right side. One day after transplantation the mice were intraperitoneally stimulated with 200µg polyI:C to induce endogenous IFN expression. After 3 hours all mice were confirmed for induced bioluminescence (Figure 26B, C). Subsequently, the mice were separated into two groups consisting of four mice (three transplants/mouse). One group received an initial dose of 200µl 0.4mg/ml doxycycline (in PBS) i.p. and then doxycycline was added to drinking water (2mg/ml doxycycline) (Figure 26A). The other group served as a control group and was treated with PBS. In the control group, the signal induced luciferase expression remained until 8 h after polyI:C addition (Figure 26B, D). However, mice who received doxycycline treatment displayed a significant decrease in reporter expression and showed a severe reduction almost down to basal levels at 8h after polyI:C (Figure 26C, E). Thus, the treatment with doxycycline after polyI:C administration decreased luciferase expression within a short period of time. The ability to interfere with ongoing IFN signalling proves the integrity of the synthetic circuit in vivo.

Taken together, this provides proof of concept that infection induced signals can be re-wired to heterologous gene (in this case luciferase) in context of mice.

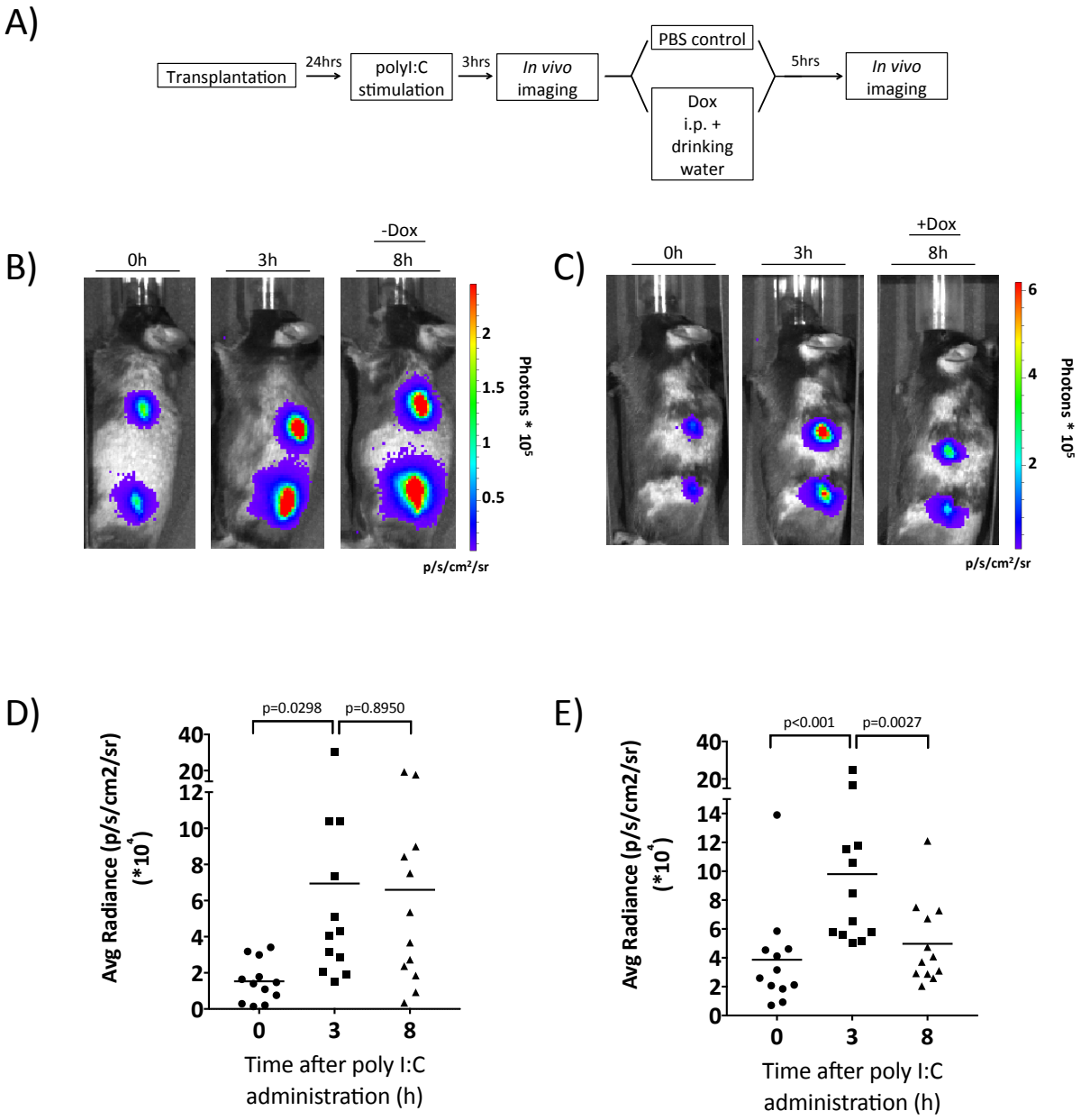

Figure 26: Doxycycline dependent repression of IFN induced luciferase expression *in vivo*.

Time line of experimental setup (A). One million DIMx2-tTA-Luc C13 cells were transplanted subcutaneously in *RAG2/IL2rg* double knockout mice. Each mouse received three cell implants in total. Two at the left side and one on the right side of the back. One day after transplantation the mice were challenged with 200µg polyI:C given intraperitoneally. Mice were imaged before and after treatment to determine luciferase expression activity as described before. After three hours mice were either left as control group treated with 200µl PBS i.p (A) or with 200µl doxycycline (2mg/ml in PBS) along with 2mg/ml doxycycline in the drinking water (C) Representative mice were shown over the course of time with respect to their treatment. Quantification of the bioluminescence signal over the time was performed with "Living Image" software. Each point represents one cell transplantation (D for non-doxycycline treatment group and E for doxycycline treatment group after three hours) (n=12).

4. Discussion

Endogenous cellular signalling pathways display one of the best orchestrated mechanisms to respond to exogenous signals. The transfer of information from the outer membrane to the nucleus and the induction of a subsequent response is a tightly regulated process that evolved and has been fine-tuned over millions of years (Pires-daSilva and Sommer, 2003).

Using naturally occurring pathways to regulate transgene expression has recently been shown to be a powerful tool to direct exogenous signals and to convert them to a desired output (Xue et al., 2017; Ye et al., 2017). Whereas classic approaches introduce reporter genes (e.g. GFP) under a specific endogenous promoter to follow promoter activity the subsequent amplification or introduction of amplification loops is not possible. Connecting synthetic regulatory units to specific endogenous signalling pathways uncouples these pathways from their natural target. By this, the input signal can be rewired to a desired output based on the respective building blocks. This is one of the main features of synthetic biology.

State of the art construction of synthetic circuit relies on the random integration of these building blocks into the host genome. In this study, the synthetic tTA transactivator was specifically targeted into the genomic Mx2 locus of murine ES cells. This gene has been shown to be specifically induced by type I and III IFN. This system offers the possibility to branch infectious/inflammatory signalling to synthetic cassettes. Due to the modularity these cassette can either consist of reporters and/or biological functional genes which can be additionally modulated by external signals. Based on the design of the synthetic circuit the initial given input signal can be amplified resulting in a distinct enhanced output signal.

The use of type I/III IFN signal as an indicator for infectious/inflammatory signalling in combination with the possibility to encompass a variety of output signals can be used to modulate this signalling in a medical orientated way. Based on the source of the input trigger (bacterial, viral, inflammatory) the output signal can be adjusted easily by uploading appropriate visualizing or counteracting synthetic cassettes.

4.1 CRISPR/Cas9 based HDR mediated targeting of the Mx locus in pluripotent and somatic cells

In order to connect the endogenous IFN signalling pathway with a synthetic circuit the tTA transactivator derived from the Tet-off system was integrated into the endogenous Mx2 locus by homologous recombination in this study. To increase the efficiency of homologous recombination, the CRISPR/Cas9 method was used to foster DNA double strand breaks and thereby induce the endogenous cellular DNA repair pathway. By co-transfer of the tTA repair template together with a vector encoding Cas9 and gRNAs directed to the Mx2 locus homology directed recombination was performed at this specific locus. Thereby the precise integration of the synthetic tTA transactivator gene in the endogenous Mx2 locus was achieved in a single transfection step. Three gRNA combinations have been used for targeting the 5´ prime and 3´ prime end of the endogenous coding sequence of the Mx2 gene. After the selection process 7 out of 15 analysed clones from these combinations could be verified as targeted with the tTA repair template. This reflects an overall homologous recombination efficiency of 47% in mES cells.

In a similar experimental setting using mouse embryonic stem cells a repair template with 1kb homology could be targeted into the Cetn1 locus with an efficiency of 8% (18/224 analysed clones) (Oji et al., 2016). In human induced pluripotent stem cells (iPSC) homology directed repair mediated knock-in with an efficiency of 20-30% has been shown (Zhang et al., 2017). Noteworthy, in this report a double cut HDR donor was used in combination with CCND1, a cyclin that is important for G1/S transition. There are also reports implementing the process of selection gaining 51-79% targeting efficiencies in hPSCs (Ding et al., 2013). These results are in line with the here obtained recombination frequencies.

The comparison of the efficiencies between different reports is hampered by the fact that not only the target locus but also the experimental setup differ between the various studies which definitely has an impact on the targeting efficiencies. Efficient delivery of the Cas9 along with the gRNAs and the repair template is a prerequisite for high targeting efficiencies. Therefore, different delivery methods can be expected to have a significant impact on the overall number of targeted clones. Viral and non-viral delivery systems have been successfully used. Usually, viral systems provide a higher transfection efficiency and deliver genetic material also in difficult to target cells (Skipper and Mikkelsen, 2015). Non-viral delivery systems like

lipofection or electroporation have been optimized for increased efficiency and have become the most used *in vitro* delivery methods in hiPSCs (Liang et al., 2015). Apart from this, the efficiency of the endogenous HDR machinery in the target cell and the architecture of the repair template with the homology regions contribute to the overall efficiency of targeting.
The targeting efficiencies achieved for the Mx2-tTA locus in mES cells are thus in the range of reported efficiencies. At this point it might be noteworthy to mention that the efficiency of targeted integration of the tTA in the Mx2 locus in ES cells was much better than a similar experiment in NIH3T3 fibroblasts. Also in NIH3T3 cells the different gRNA combinations as well as the repair template were introduced via lipofection. Although eGFP positive cells could be obtained (indicating successful Cas9 delivery) the subsequent analysis did not reveal cell clones with correct integration of the tTA in the Mx2 locus (data not shown). From 200 clones which were screened not a single clone displayed correct functional integration of the tTA.
The difference between the targeting efficiency in embryonic stem cells and NIH3T3 might have several reasons. Cells have evolved different mechanisms to counteract DNA damages to detect and promote their repair (Harper and Elledge, 2007). Collectively these processes are termed DNA damage response (DDR). Cas9 introduces double strand breaks into the genome which can be repaired by two different cellular repair pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR) (Brandsma and Gent, 2012). Both repair pathways utilize a different set of proteins for recognition of the lesion and the subsequent repair mechanism and are active at different stages of the cell cycle (Rothkamm et al., 2003). There is evidence that embryonic stem cells favour the use of the homologous recombination repair (HRR) pathway upon DNA double strand breaks. This is supported by different observations. In HRR, DNA double strand breaks are repaired by the use of a homologous template. This is usually the sister chromatid or homologous chromosomes. As these templates are mainly accessible during interphase of mitosis HRR is mainly active in late S or G2 phase (Haber, 2000). Embryonic stem cells lack a G1 checkpoint and have a very short cell cycle in G1 and G2 phase whereas 75% of cell cycle duration is spent in the S-phase (Savatier et al., 2002). As a consequence HDR is favoured over NHEJ in embryonic stem cells. The correct maintenance of the genetic information in embryonic stem cells is a crucial aspect. This might be more obvious with regard to the fact that these cells give rise to progenitor cells where appropriate performance has to be ensured. Mutations

which arise from NHEJ would be passed to these progenitors which could lead to their functional impairment. In contrast HDR is limited in somatic cells.

Efforts to elucidate the role of the proteins involved in HRR have been hampered by the fact that since genetic modification of the respective genes usually results in early embryonic lethality (Friedberg and Meira, 2006). Analysis of Rad51, the key player in HRR, revealed a 20-fold higher protein level in ES cells compared to mouse embryonic fibroblasts (MEFs) where NHEJ predominates (Tichy and Stambrook, 2008). Also other proteins involved in HRR are elevated in ES cells compared to MEFs (Tichy et al., 2010). These include Rad52 and Rad54. Vice versa, the abundance of enzymes involved in NHEJ (Ku70, Ku80, DNA ligase IV) is decreased in ES cells and increased in somatic cells. With regard to NHEJ the subsequent DNA strand religation after processing is mediated by DNA ligase IV (Grawunder et al., 1997). In ES cells the half-life of this ligase is low but when stem cells are subjected to differentiation the protein levels of DNA ligase IV increase along with the capacity of NHEJ as the preferred DNA repair mechanism. Strategies have been obtained to inhibit NHEJ in somatic cells and thereby increasing the efficiency of precise genome editing via HDR (Maruyama et al., 2015). Vice versa the HDR enhancer RS-1 a stimulator of Rad51 has been shown to improve gene editing via HDR (Song et al., 2016). Inhibition of NHEJ or enhancing HDR could be useful to target the Mx2 locus even in cells that have lower HR frequencies such as NIH3T3 fibroblasts. Additionally, further optimization using different DNA delivery methods (e.g. electroporation) might be useful. With regard to the screening process to identify a functional tTA clone the use of a reporter cell line (e.g pTet-RFP) without tTA might be of help. Here, the tTA could be targeted into the Mx2 locus and the selection process would provide clones with an integrated selection cassette. To identify functional clones the addition of IFN and subsequent cell sorting approach for RFP positive cells would identify functional targeted clones which could transmit IFN signalling to a cellular reporter.

4.2 Differentiation state dependent IFN response

ESMx2-tTA cells in which the synthetic transactivator tTA is controlled by the endogenous Mx2 promoter displayed a strictly IFN dependent induction profile similar to the endogenous Mx2 gene both in the stem cell state and upon differentiation. Stimulation with IFN resulted in comparable induction rates of 4.3 for Mx2 mRNA transcripts and 4.9 for tTA transcripts in the

ES cell state. Remarkably, upon differentiation the induction rates of both genes were markedly enhanced reaching fold induction levels of 44 for Mx2 and 62 for tTA.

The impaired IFN response in the ES cell state is a largely uncharacterized property of ES cells that seems to be unique for this cell type. Within the last years several studies have lighted up the development of innate immunity and ESC physiology. The attenuated response in both human and murine ES cells to IFN became evident and is still under investigation.

Human pluripotent cells (hESCs and hiPSCs) fail to express ISGs in response to dsRNA as a consequence of an underdeveloped innate immune response (Chen et al., 2010). This is not due to a lack of signalling molecules in the IFN pathway because these are expressed although at low levels. The malfunction to IFN seems to be mediated by high expression of suppressor of cytokine signalling 1 (SOCS1) (Hong and Carmichael, 2013). Upon differentiation of hESCs the expression level of SOCS1 is decreased but upregulated rapidly after IFN stimulation. In the undifferentiated state, SOCS1 negatively regulates the JAK/STAT signalling pathway to limit IFN response (Alexander, 2002). Noteworthy, SOCS1 does not belong to the family of ISGs as it is also induced by other cytokine signals (Fujimoto and Naka, 2010). SOCS1 has been reported to interact with critical phosphotyrosine residues located in the JAK catalytic loop (Yasukawa et al., 1999) but also associated with phoshotyrosine residues of IFNAR1 receptor units in a JAK-independent manner have been shown.

In contrast to their human counterparts mouse embryonic stem cells are able to respond to IFN-α and IFN-β leading to induction of ISG and mediating an antiviral effect (Whyatt et al., 1993). However, the response to IFN is much weaker compared to that of fibroblasts (Wang et al., 2014). In contrast to the regulatory mechanisms present in human ESCs SOCS1 seems not be the major repressor limiting IFN response. SOCS1 mRNA expression in mESCs is similar to that of mouse fibroblasts and siRNA targeting SOCS1 had no effect on ISG induction. The underlying mechanism is so far not resolved (Guo et al., 2015b).

Mouse fibroblasts reprogrammed to iPSCs lose their antiviral response (Chen et al., 2012). It seems that there is an inverse relationship between antiviral response and pluripotency itself. This may have several reasons. IFN has pleiotropic effects e.g. it inhibits proliferation (Hertzog et al., 1994). As pluripotent cells undergo rapid cycles of cell division the anti-proliferative effect of IFN could decrease or inhibit cell cycle progression of pluripotent cells. In line with this is the observation that pluripotent cells are primed to undergo rapid apoptosis (Dumitru et al., 2012). It has been shown that activation of the STAT signalling pathway can cause

apoptosis (Chin et al., 1997). Additionally, it was shown that IFN stimulated the process of differentiation (Hertzog et al., 1994). This would in turn perturb the cell state and induce unwanted reprogramming of pluripotent cells.

It is not clear whether the lack of a robust antiviral response in pluripotent cells is needed to maintain stem cell characteristics or if the stem cell state restricts the development of antiviral mechanisms (Guo et al., 2015a).

Although the embryonic stem cells display an underdeveloped IFN system the cell state offers the opportunity to differentiate them into different types of cells and thereby establishing a fully functional IFN pathway characteristic for the specific cell type. This system offers great potential as virtually any cell type of interest could be obtained by *in vitro* differentiation harbouring the recombined Mx2 locus.

4.3. The Mx2-tTA system as a novel synthetic circuit to rewire infection/inflammation signals

In this study, a synthetic network was established by integration the tTA transactivator into the Mx2 locus. By adding tTA dependent expression modules, IFN signalling was rewired to reporter gene expression or expression of mIL10 as an effector. So far classical approaches tend to integrate genes of interest (e.g. reporter) directly into the desired locus. In contrast to this the synthetic tTA approach has a number of advantages over these state of the art design. The great potential of the circuit is inherent in the ability to transfer IFN signalling to synthetic cassettes and based on the modularity of the system the ability to introduce alterations of the circuit in a building set principle. This offers not only the possibility to rewire IFN signalling to a variety of genes of interest but also to introduce positive feedback loops amplifying the initial input signal. The circuit itself is self-limitig, depends on pathophysiological IFN concentrations and can be easily controlled external by the application of doxycycline for fine tuning or complete shut-down of the circuit.

4.3.1 Connecting IFN signalling to synthetic cassettes

To evaluate the performance of the synthetic circuit, initially a luciferase reporter was stably introduced into ESMx2-tTA cells. The expression of tTA could be transmitted to luciferase expression in an IFN dependent fashion. This was true for the ES cell state as well upon differentiation. In agreement with previous reports (Wang et al., 2014) the overall luciferase activity in presence of IFN was moderate in ES cells compared to activity after differentiation which was 14 times higher. The different activity of luciferase after IFN stimulation was found to be the result of a difference in tTA expression in these cell states. Together, an authentic, cell state dependent transmission of IFN signalling to reporter expression is achieved within the ESMx2tTA system.

The treatment of the IFN stimulated ESMx2-tTA cells with doxycycline reduced luciferase activity to the levels that were detected from these cells in absence of IFN. This was observed both for the stem cell state and after differentiation. This demonstrates that in ESMx2-tTA cells tTA expression is tightly regulated by IFN and no leaky expression is mediated from the Mx2 promoter. These results are in line with the previously reported tight regulation of the Mx2 promoter (Asano et al., 2003). Furthermore, the efficient repression to basal levels revealed that luciferase expression was exclusively tTA dependent which excludes unspecific induction of luciferase. Together, the results show that ESMx2-tTA cells express tTA in a strictly IFN dependent manner and allow tight regulation of tTA dependent genes.

4.3.2. Amplification of reporter signal using autoregulated circuits

The integration of a synthetic transactivator to endogenous signalling cascades offers the ability to connect its expression to a variety of transactivator dependent modules. In this study, it was first connected to a luciferase reporter to monitor its IFN dependent expression and temporal resolution. To evaluate if the initial derived reporter signal could be amplified a positive feedback loop was implemented in ESMx2-tTA embryonic stem cells, resulting in ESMx2-tTA-Autoluc cells. This loop was realized by stable integration of an expression cassette in which the tTA transactivator was controlled by the tTA dependent promoter.

Autoregulated cassettes depend on a basal expression of the tTA transactivator from Tet-operator sequences in the repressed state. In the induced state, the transactivator

induces its own expression and additionally the expression of e.g. reporter genes (Shockett et al., 1995; May et al., 2008) Thereby the signal is amplified in comparison to systems where tTA is expressed at constant levels (e.g. from constitutive promoters). These properties of autoregulated cassettes could explain the high basal activity of luciferase in absence of IFN in ESMx2-tTA-Autoluc cells in which the positive feedback loop was implemented. In line with this is the observation that expression was tTA dependent as treatment with doxycycline showed. Unexpectedly, however, the addition of IFN had no impact on luciferase expression in the stem cell state. As luciferase levels are already highly elevated in absence of IFN the minor effect of IFN stimulation of ES cells might not lead to enhanced luciferase expression account.

Upon differentiation of the ESMx2-tTA-Autoluc cells an increase in luciferase expression could be detected which could be attributed to the improved IFN signalling pathway of differentiated cells. In contrast to the ES cell state there was IFN dependent luciferase expression detectable indicating that the synthetic cassettes are integrated in a functional way. The overall mean luciferase activity could be enhanced in comparison to the ESMx2-tTA-Luc cells. Notably, also here there was still an elevated basal level detectable derived from tTA activity. The range of luciferase expression was reduced to that observed in the ES cell state and could also here be attributed to leaky tTA expression from the autoregulated unit.

4.4 Epigenetic silencing of Tet-cassettes in ES cells – a challenge for synthetic biology applications

Upon differentiation of long-term cultured ESMx2-tTA-Luc cells a dramatic decline in the overall luciferase expression was observed. Along with a decrease in the basal levels a reduction in the absolute reporter expression in IFN stimulated conditions was detected so that there was no significant difference in the relative induction rates between early and late passage. The expression of the luciferase reporter is dependent on a) the expression of the IFN induced tTA transactivator and b) the subsequent binding of the transactivator to Tet-operator (*TetO*) sequences driving luciferase expression. The latter could be influenced by a repressive chromatin structure reducing chromatin accessibility and impeding DNA binding of the transactivator (Loew et al., 2010).

The Tet-system has been used successfully in a plethora of different studies. These include disease models (Traykova-Brauch et al., 2008), drug screening (Gonzalez-Nicolini and Fussenegger, 2005) and RNA interference (Zhang et al., 2007) to mention some. The system works efficiently not only in a variety of cultured cells of different origin (Bornkamm et al., 2005) but also in whole organisms like Dictyostelium, Drosophila, amphibian and mammals. (Berger and Bujard, 2004). Although the the system was described already 25 years ago the different elements are still further optimized with regard to their functionality (Müller et al., 2015; Roney et al., 2016). This results in the high applicability and strength of the system.

However, there are some reports that show that the tTA transactivator-responsive promoters (*Tet$_O$*) become functionally silenced in certain conditions/experimental settings (Kues et al., 2006; Zhu et al., 2007). Yu et al., demonstrate a loss of transgene expression from *Tet$_O$* cassettes in cancer cells which could be either restored by application of a histone deacetylase inhibitor or maintained by continuous induction of the transgene (Yu et al., 2016). Similarly, Zhu et al., point out that epigenetic control mechanisms in certain cell types of the brain are involved in transactivator dependent reporter activation (Zhu et al., 2007). In a more comparable approach to this work Kues and coworkers could inversely correlate the transgene expression pattern with the methylation status of the Tet promoter (Kues et al., 2006).

Treatment of the ESMx2-tTA-Luc cells with the DNA methyltransferase inhibitor decitabine during the differentiation process resulted in an increased overall luciferase expression compared to untreated cells. The inhibition of DNA methylation increased basal luciferase expression as well as luciferase expression upon IFN stimulation. This suggests that the Tet-cassettes get silenced upon long-term cultivation of ES cells and subsequent differentiation. The decrease in basal expression influences in turn the overall luciferase expression in presence of IFN. As the luciferase reporter construct was randomly integrated into the genome the local chromosomal status might influence its gene expression (Whitelaw and Martin, 2001). Endogenous regulatory elements which are in close proximity and the chromatin structure might have an effect on transgene expression which is referred to as position effect. This is often associated with limited level of transgene expression (Wilson et al., 1990). In the case of random integration the target site is not predictable in contrast to targeted integration. Thereby the effects, positive or negative, on target gene expression are unpredictable. In our own laboratory the silencing of the Tet-cassettes was systematically investigated in more detail in mouse embryonic stem cells, thereby particularly addressing the

impact of the chromosomal surrounding of the inducible cassettes. To study the expression properties of *Tet* cassettes, the Rosa26 locus as an open ubiquitously expressed chromosomal site in embryonic stem cells was chosen. The Rosa26 locus has been used in a variety of different studies and has been proven to verify stable expression in mice (Sandhu et al., 2011). In the study described by Gödecke and coworkers integration of Tet-cassettes driving reporter expression within this locus led to a subsequent partial or even complete silencing of inducible reporter expression which was shown to be accompanied with massive methylation of the Tet promoter. This was true for the cultivation of embryonic stem cells was well as for the process of differentiation. Noteworthy, the flanking endogenous Rosa26 and ThumbD3 promoters were not affected. This indicates that similar to the observation in this thesis in embryonic stem Tet-cassettes cells are negatively affected upon long term culture and differentiation. In both cases the open chromosomal state at this site does not prevent silencing mediated partially by DNA methylation. A possible mechanism to reverse methylation of the Tet promoter is to specifically guide DNA demethylation enzymes to target DNA sequences. A fusion protein consisting of the catalytic domain of Tet1 and of the DNA binding domain (*TetR*) of the tTA transactivator was used, designated TetR-rtTA-Tet1c. Tet1 is known to initiate the first step of DNA demethylation. The TetR-rtTA-Tet1c fusion protein was able to decrease the methylation level of the Tet promoter and thereby restoring reporter expression of previously silenced circuits (Gödecke et al., 2017).

Targeting the Tet1 catalytic domain to specific sites within the genome has been shown to be a powerful genetic tool to modify the epigenetic state. The genome editing systems TALEN (transcription activator-like effector nuclease) or CRISPR/Cas9 have been adapted to target DNA demethylation enzymes specifically to DNA target sequences and restore gene expression (Maeder et al., 2013; Vojta et al., 2016). Similar to the TetR-rtTA-Tet1c approach these fusion proteins can be used to unravel the underlying mechanism of site specific DNA methylation in a genomic dependent context.

The TetR-rtTA-Tet1c fusion protein was not only able to initiate demethylation of the Tet promoter but also to initiate expression of the reporter. This indicates that the transcriptional activator domain of the tTA fusion protein was still functional. Integration of a TetR-tTA-Tet1c transcriptional activator into the Mx2 locus might be a possibility to overcome the silencing mechanism during long term cultivation of the ES cells and additionally could be used as a

synthetic transcription factor. This might also be true for TALEN or dCas9 (catalytic inactive Cas9) fusion proteins.

Another possibility to overcome the obstacle of silencing would be to introduce epigenetic regulatory elements such as matrix attachment regions (MARs) (Bell et al., 1999), ubiquitous chromatin opening elements (UCOEs) (Lindahl Allen and Antoniou, 2007) or chicken cHS4 insulators (Rincon-Arona et al., 2007). All these elements influence the stability of transgene expression by an epigenetic signature. However, it remains to be shown if and to which extent they can protect the Tet cassettes in embryonic stem cells.

There are two publications showing that silencing of Tet-cassettes could be inhibited by keeping the Tet cassettes induced (Zhu et al., 2007; Yu et al., 2016). The overall silencing mechanisms in both reports are addressed to the lack of inducibility of transgenes when transgenic cells are kept uninduced over a long period. The authors suggest that transactivator binding to Tet-cassettes maintains promoter accessibility and thereby prevents silencing. With regard to inducible promoters like Mx2 a constant activation of this promoter might be affected by negative feedback loops decreasing IFN signalling in order to prevent overactivation of infectious signalling. Furthermore, the constant administration of IFN itself would affect the cellular state e.g. inhibition of translation, induction of apoptosis (Chawla-Sarkar et al., 2003).

4.5 Maintenance and improvement of IFN dependent luciferase expression after immortalization – Mx2-tTA embryonic stem cells as a resource for any cell types of interest

Two essential properties are characteristics of embryonic stem cells: the capacity of self-renewal and the ability to differentiate into different cell-type specific progenitors. Latter is accompanied by the programming of cells resulting in the activation or repression of specific genes. This process is mainly mediated by tight control of epigenetic modulation. The negative epigenetic regulation of the Tet-promoter impairs the establishment of a robust regulatory circuit in the ES cells. Nevertheless, for future applications the establishment of cells of specific origins might be favourable.

As an alternative to the ES cell based reporter system in this study, also the cell line DIMx2-tTA was evaluated. These cells were generated from ESMx2-tTA cells upon differentiation and subsequent immortalisation. Upon introduction of the tTA dependent

reporter luciferase IFN signalling could be stably transmitted in a tTA dependent manner. Importantly, in this cell line, expression was stable over time suggesting that the Tet cassettes are not impaired by silencing. Furthermore, the DIMx2-tTA-Luc cells displayed an improved phenotype with regard to IFN sensitivity, i.e. less IFN levels could already induce reporter expression.

Besides the recent reports that embryonic stem cells have an impaired IFN signalling also other tissue and cell-type specific responses to IFN have been reported before. The type I interferon receptor is ubiquitously expressed while the type III receptor expression is restricted to the epithelium of mucosal surfaces and to a few other cell types (Sommereyns et al., 2008). Furthermore, the cell polarization state and epigenetic status have been shown to have an impact on IFN signalling (Bhushal et al., 2017). Additionally, the differential activation of STATs in different cell types might contribute to a different induction of ISGs (van Boxel-Dezaire, Anette H H et al., 2006). All these factors influence the responsiveness of a specific cell type to IFN. In the DIMx2-tTA-Luc cell line an interaction of these or other factors might contribute to the improved sensitivity.

It has been reported previously that the simian virus 40 T antigen upregulates many interferon-stimulated genes by activating STAT-1 in absence of interferon (Forero et al., 2014). Activation of STAT-1 may have a direct effect on Mx2 itself thereby increasing basal expression. This could have an impact on the basal expression of the tTA system in the DIMx2-tTA cells which were immortalized with this multifunctional oncoprotein. However, such an effect was not observed. Rather, the basal rates of luciferase activity were similar to these obtained upon differentiation of the engineered ES cells before immortalization and between individual reporter cell populations. In line with this the basal luciferase activity could not be further reduced in presence of doxycycline. The absence of an elevated IFN response in these cells could be attributed to the amount of T antigen which is sufficient for cellular transformation but insufficient to initiate innate immune response. Furthermore, it might be that the activation of signalling pathways after SV40 transformation is cell type dependent and thereby also the consequences might differ from cell type to cell type (Cantalupo et al., 2009).

The functional integration of synthetic cassettes after differentiation (and immortalization) can be used as a tool to equip specific cell types with specific circuits. The ESMx2-tTA cell line can be used as a “master” cell line which can be differentiated into the desired cell type.

Afterwards the integration of synthetic cassettes offers the possibility to equip these cells with a specific circuit.

4.8 BAC vectors as a tool to amplify MX-tTA signal converters and increase output signals

The synthetic transactivator driven by the endogenous Mx2 promoter in DIMx2-tTA-Luc cells proved its functionality with respect to the detection of IFN and transfer this signal to a quantifiable reporter protein. Within these cells one copy of transactivator was sufficient to induce luciferase expression significantly. However, with this approach the maximal number of possible cassette integration sites is n=2 given the fact that the endogenous Mx locus is located on two chromosomes. In an approach to increase the overall amount of transactivator molecules and keeping the Mx2 genomic contexts bacterial artificial chromosomes (BAC-Mx2-tTA) were designed and evaluated.

BACs have been shown to reflect the expression of endogenous genes after transfer in heterologous cell contexts. They comprise few hundred kb of sequence information of a locus of interest. Thereby putative cis-regulatory elements are included that mediate this context dependent expression. Upon targeted integration of transgenes by *in vitro* recombineering, BACs can reflect authentic expression. Apart from generation of transgenic organisms they are also suitable to identify possible regulatory elements within flanking regions of genes (Mortlock et al., 2003; Smith, 2008). This also explains why the expression of BAC encoded transgenes is frequently position- independent as long the physical intact BAC is integrated (Rival-Gervier et al., 2002).

In this study, the tTA gene was integrated into the BAC-Mx2 by *in vitro* recombineering. Upon transfection of NIH3T3 cells clones were isolated. The initial screening approach displayed that not all selected BAC-Mx2-tTA clones displayed the same tTA activity. This might indicate BAC fragmentation during the process of genetic manipulation and the failure of full-length BAC integration in some clones. Similar observations have been reported before and make clonal screening necessary to identify those cell clones which show integration of an intact BAC vector resulting in authentic expression (Rostovskaya et al., 2012; Gillen et al., 2013) .

The best inducible clone was selected for subsequent integration of the Tet-luc reporter. The high degree of basal reporter expression in all seven BAC-Mx2-tTA-Luc clones could be addressed to an elevated basal activity of the transactivator in absence of IFN. This was proven

by the reduction of expression in presence of doxycycline. This degree of high basal activity of the transactivator within these cells was not observed in the DIMx2-tTA-Luc cells suggesting that it was a consequence of the BAC transfer rather than a property of the Mx2 locus. The non-authentic activation of tTA might be due to a rearrangement and/or insertion of fragmented BAC vector sequences into the cellular genome as mentioned earlier in this chapter. Such fragmentation can occur upon physical constraints such as shear stress induced during handling of BAC vectors during transfection (Antoch et al., 1997; Kaufman et al., 1999). Thereby fragmented BACs integrates in the host genome which do not reflect endogenous gene expression (Giraldo and Montoliu, 2001).

IFN stimulation resulted in a mean luciferase expression of 10^8RLU/mg. Compared to the DIMx2-tTA-Luc C13 system this is highly elevated and might indicate that a higher tTA transgene copy number is integrated in BAC-Mx2-tTA compared to the DIMx2-tTA system.

4.9 Conversion of pro-inflammatory signals to anti-inflammatory cues via the synthetic circuit

One aim of this study was to create a cellular system capable of sensing infection and inflammation signals and transmitting these to synthetic promoters. The transmission itself was mediated by the tTA transactivator which was induced upon sensing IFN. Luciferase expression was the read-out of choice to validate the performance of the circuit.

Infection and inflammation are processes closely related to each other. However, inflammatory disorders like atherosclerosis or allergies are not associated with any kind of infection. Nevertheless, most infections cause the production of cytokines by the innate immune system at the site of infection. This in turn leads to the recruitment of neutrophils and mononuclear cells (monocytes, macrophages). These are consequences of the initial infection but refer to the process of inflammation. A key player in the inflammatory process is IL10. IL10 is produced by many different myeloid and lymphoid cells where macrophages are the main source of IL10. The key role of IL10 during infection is the downregulation of pro-inflammatory processes before pathology occurs. To do so, IL10 suppresses macrophage and dendritic cell function and limits effector responses of T cells (Th1 and Th2) (Bashyam, 2007).

Due to the multiplicity of IL10 functions it was selected as an anti-inflammatory mediator and incorporated into the Mx2-tTA circuit. Expression analysis revealed an IFN dependent induction of IL10 in both DIMx2-tTA-mIL10 and BAC-Mx2-tTA-mIL10 cell lines.

In humans, physiological levels of IL10 are in the range between 2-13pg/ml (Kleiner et al., 2013). Upon infection levels up to 280pg/ml can be reached (Medina et al., 2011). Stimulation of macrophages with prostaglandin 2, a principle mediator of inflammation, resulted in the secretion of IL10 in the range of 170pg/ml (Ylöstalo et al., 2012).

Since the BAC-Mx2-tTA cells showed highest levels of the reporter expression, the BAC-Mx2-tTA-mIL10 cells were used to determine the amount of secreted mIL10 by ELISA in the culture supernatant after stimulation with IFN-β. A 3fold induction in presence of IFN could be detected with maximal mIL10 levels at 320pg/ml after 48 hours. A main aim in synthetic biology is the engineering of cellular systems with so far absent biological features. Synthetic circuits are built in a way that cells gain the ability to respond a trigger in a new genetically encoded ability. Whereas wildtype NIH3T3 fibroblasts lack the ability to secret mIL10 upon IFN-β stimulation the NIH3T3 BAC-Mx2-tTA-mIL10 fibroblast reach concentrations of mIL10 comparable to physiological levels upon infection. The presence of doxycycline reduced mIL10 to basal levels. This proved that mIL10 expression was transactivator dependent and INF-β had no influence on endogenous mIL10 expression.

In a recent study a synthetic circuit was described using mIL10 as an anti-inflammatory cytokine to counteract the phenotypic manifestation of psoriasis (Schukur et al., 2015). In this study HEK293T cells were engineered to secret mIL10 upon the presence of inflammatory cytokines typical for psoriasis. Alginate encapsulated engineered cells were used to restore symptoms of the skin disease. The *in vivo* mIL10 levels cytokines levels obtained from blood displayed a 2-3fold higher mIL10 level than control cells. This displays that minimal changes in the concentration of mIL10 have a high impact on the anti-inflammatory response mediated in this model. Based on these results a tight regulation might be of more advantage that a high induction of mIL10. The BAC-Mx2-tTA-mIL10 cells engineered in this work display this regulatory feature with regard to IFN dependent mIL10 production. Transplantation of these cells to sense and counteract inflammatory states (e.g. bacterial infection/implantation) *in vivo* would give more insights into the ability of these cells to restore physiological conditions.

4.10 Sensing infection *in vivo* with synthetic modified cells

Treatment of cells containing the synthetic circuit *in vitro* already proved their functionality with regard to rewire IFN signalling to a reporter molecule. For future applications the circuit should be used to detect infectious derived signals *in vivo* and transmit these signals to specific genes of interest.

Both engineered cellular systems (DIMx2-tTA-Luc and BAC-Mx2-tTA-Luc) could transmit IFN signalling derived from the endogenous system upon polyI:C stimulation to the luciferase reporter. Additionally, both systems responded within a narrow time window (3 hours) which highlights the applicability of such cells for the induction of immediate biological functions. The intervention of infection/inflammation has to be timely orchestrated. An intervention circuit should counteract pathogen presence as early as possible, in particular before onset of e.g biofilm formation. In bacterial infections dissemination takes place within several hours (Barman et al., 2011). *In vivo* evaluation of biofilm-related infections revealed that prolonged (21 days) combination therapy is beneficial in treatment. The system established here could serve as a prosthetic network not only sensing bacterial infection but also establishing a counteracting response before establishment of a serious hard to treat infection. This could either be mediated by the providing IFN mediated expression of immunomodulatory genes that strengthen the host defense or of anti-bacterial genes thereby mediating a direct anti-bacterial effect already at early time points after infection. The integration of a synthetic transcription factor under the Mx2 promoter also offers the huge advantage of combining immunomodulatory genes with anti-bacterial genes within one synthetic circuit.

In the cells established in this study, the tTA transactivator activity can be downregulated in the presence of doxycycline *in vitro*. One important question of the system was if doxycycline dependent repression of IFN induced luciferase reporter expression could also be achieved *in vivo*. Therefore, DIMx2-tTA-Luc C13 cells were subcutaneously transplanted into immunocompromised *RAG/IL2rg* double knock out mice and challenged with polyI:C to stimulate endogenous IFN expression. The intraperitoneal administration of doxycycline was sufficient to downregulate luciferase expression in a narrow time window. The external regulation of the circuit even in ongoing IFN signalling offers great potential for future applications. In conventional Tet-systems doxycycline mediates an ON or OFF state of gene expression and is thereby used to modulate subsequent cellular effects (Schönig et al., 2002;

Hong et al., 2007). In the system established here expression of the tTA transactivotor is mediated by IFN signalling and doxycycline independent in the first place. Here, doxycycline can be used on a secondary control level and would be used as a kind of kill-switch to shut transgene expression off within a short period. In combination of the circuit with therapeutic agents this could be used to control unwanted not foreseeable responses and to prevent a prolonged induction. This offers a beneficial and temporally highly controllable kill-switch option for uncontrolled gene expression.

For future applications it will be of interest to prove that the transmission of IFN dependent tTA activity to e.g. a biological factor (mIL10) activates a biological response *in vivo*. In particular, it has to be evaluated if sufficient amounts of the biological factor IL10 are induced and secreted. In case the amounts are not sufficient there are various options to enhance IL10 secretion. The integration of amplification loops as proven in the ESMx2-tTA-Autoluc cells is a possibility to increase the cellular amount of tTA molecules and subsequently IL10 secretion.

4.11 Comparison between targeted single-copy and random multi-copy integration

Comparing the DIMx2-tTA-Luc C13 with BAC-Mx2-tTA-Luc with regard to their *in vivo* performance upon pathophysiological IFN concentrations both systems displayed their functionality. However each of the systems offers different advantages. The DIMx2-tTA-Luc C13 system in contrast to BAC-Mx2-tTA-Luc shows a better inducibility whereas the BAC-Mx2-tTA-Luc systems seems to have a general higher expression rate. In case of the BAC modified cells this is due to a general higher basal tTA activity. It is suggested that the specific features of the two systems can be used to fulfil different aspects: When tight regulation is needed the DIMx2-tTA systems will be favourable. This system offers almost no basal tTA expression *in vitro* and is highly inducible. The tight regulation would rule out detrimental effects of leaky expression. In contrast, if a high dosage of a protein or mRNA is needed the BAC-Mx2-tTA systems will be of more advantage. Although the maximal mean induction fold was not comparable with the DIMx2-tTA system the overall luciferase expression of the system is highly increased. Thereby, high expression rates after IFN stimulation are achieved. It should be noted that many biological functions require elevated expression level; below a certain threshold, gene expression does not lead to the change in phenotype. This was e.g. shown for a synthetic (positive feedback) cassette controlling SV40 T-Antigen, where even

considerable basal levels did not result in proliferation of cells (May et al., 2010). Depending on future application one need to carefully consider which system to use with regard to the circuit which should be implemented.

The main difference between the DIMx-tTA-Luc and BAC-Mx2-tTA-Luc cells is their copy number of tTA gene integration. The DIMx2-tTA-Luc cells are derived from the ESMx2-tTA cells which have been established via homologous recombination. Thereby the tTA transactivator is integrated into the genomic location of the endogenous Mx2 gene which was subsequently replaced at least for one allele. In contrast the BAC-Mx2-tTA-Luc cells were generated via *in vitro* BAC recombineering and subsequently transfected into NIH3T3 fibroblasts. Here, a random integration of the several BACs into the genome took place. There are several reports that investigated the amount of copy number integration and proved a positive correlation with transgene expression (Chandler et al., 2007; Menezes et al., 2009).

Furthermore, future applications may be based on multiple interconnected building blocks. Here, an IFN amplification loop could be coupled to tTA activity. The IFN dependent expression of IFN from synthetic promoters could be used as auto- and paracrine intrinsic positive feedback loop. This circuit would produce its own inducer molecule. It might be of interest how these circuits behave with regard to underlying endogenous negative feedback loops.

5. Outlook

Translational research is getting more and more into focus. In the field of synthetic biology, the knowledge of basic molecular biology is combined to generate synthetic circuits used for biomedical applications. There are first examples where a pathophysiological metabolic state is sensed and rewired to a physiological state by specifically engineered cell systems. Ye et al., established a cellular system and reverted the insulin-resistance syndrome in a self-adjustable manner (Yu et al., 2016). The inability to use insulin efficiently is counteracted by the insulin stimulated expression of adiponectin which sensitizes the cells to insulin (Kadowaki et al., 2006) and has been shown to be a promising candidate for treatment of type II diabetes (Yamauchi et al., 2003). A similar approach was used to decline pathophysiological urate levels in a mouse model (Kemmer et al., 2010). Apart from the application in metabolic disorders synthetic circuits also have been implemented in the prevention of liver injury and subsequent organ failure (Bai et al., 2016).

5.1 Synthetic Immunology

In the field of immunology synthetic biology inspired cellular engineering is rapidly growing. Synthetic T cell receptor like chimeric antigen receptors (CARs) or antibody-coupled T cell receptor are just two examples (Roybal et al., 2016; Morsut et al., 2016). Apart from the specificity to a certain antigen the current generation of these cells combine intracellular signalling modules both from the TCR and co-stimulatory receptors (Wu et al., 2015).

The circuit established in this work proved the concept that infection associated signals can be rewired to heterologous effectors or reporters based on synthetic biology principles. This offers the possibility to engineer cells which mediate an anti-infectious or anti-inflammatory response *in vivo*.

A central part in the regulation of inflammation are macrophages. They contribute to the formation and stabilization of plaques in atherosclerosis (Bobryshev et al., 2016), insulin resistance, (Heilbronn and Campbell, 2008) or cancer progression (Nielsen and Schmid, 2017). Due to their mobility these cells offer great potential to locally accumulate at specific sites within the body (e.g. sides of inflammation). An increased number of macrophages at a specific site would also in turn mean that their produced gene products accumulate at these

sites. It has been shown that engineered macrophages expressing IFN-α infiltrate tumor tissues in a xenograft mouse model and stimulate immune cells thereby inhibiting breast cancer progression (Escobar et al., 2014). A similar approach could be used with the established Mx2-tTA cellular system. ESMx2-tTA cells could be differentiated to macrophages and used as a delivery vehicle to express IFN-α at the tumor site (Lachmann et al., 2015). The induction of the system would be mediated by IFN itself that is induced by DNA derived from necrotic tumor cells as recently reported (Fuertes et al., 2013). Although IFN has been used in cancer treatment the systemic administration had several side effects (Sleiifer et al., 2005). With the targeted gene circuit uploaded in macrophages a local IFN induction would rule out side effect to other organs. Furthermore IFN- α would not only inhibit cancer proliferation but also act as an amplification loop which stimulates in a positive feedback fashion its own expression.

Tumor associated macrophages (TAMs) contribute in high numbers to the tumor microenvironment (TEM). TAMs have been associated with the “alternative activated” M2 phenotype promoting anti-inflammatory and pro-tumorigenic functions (Biswas and Mantovani, 2010). On the other extreme side of their phenotype they can also be polarized in a M1 like phenotype (classically activated) with a pro-inflammatory and anti-tumorigenic phenotype. Re-education of these TAMs from a M2 to M1 state in the TEM is a major therapeutic aim and has been shown to dependent mainly on TNF-α (Shime et al., 2012). The integration of a Mx2-tTA-TNF-α circuit into myeloid cells could be used to target the TEM and make use of endogenous cells to target the tumor by reverting their phenotype.

A similar approach targeting the cancer microenvironment could be based on AND GATE logic. Here an additional second signal would be crucial to induce the full therapeutic impact of the circuit. Myeloid derived suppressor cells and tumor associated macrophages secrete interleukin 10 (IL-10), vascular endothelial growth factor (VEGF) or nitric oxide synthase (NOS) in order to protect cancer cells from the immune system. This established microenvironment makes therapeutic intervention ineffective in most cases. The endogenous signals derived from the immune cells (VEGF, NOS) could be used as a second signal necessary to induce the circuit. By this it would be guaranteed that the circuit is activated at the site where the cancer is located due to the dual input signal needed. For therapeutic intervention these systems could be designed to modulate the microenvironment making it more vulnerable for

chemotherapeutics or other anti-cancer drugs. Additionally the perturbation of the TEM could enhance the endogenous immune system with regard to tumor recognition and eradication.

5.2 Targeted delivery of therapeutic components

Apart from the direct modulation or intervention future cellular systems could also deliver a so-called cargo. This cargo could be composed of anti-cancer drugs which are specifically released on the site of cancer growth. Cellular systems could be loaded with the cargo. By being attracted to the site of cancer growth due to the inflammatory signals the second cancer specific signal could be used to induce the specific release of the cargo and thereby the drug itself. This would make therapeutic interventions more effective because the used therapeutics would be delivered at the site where they are needed. This would reduce the required dose and avoid the systemic drug administration and possible negative side effects.
The cargo which is delivered by the cells could also be based on nanotechnology. Gold nanoparticles are used as novel agents for cancer therapy. They serve not only as drug carriers but can also be utilized for thermal therapy (Abadeer and Murphy, 2016). Loading the engineered cells with gold nanoparticles or other metal particles could be used to induce hyperthermia at the cancer site once they accumulate there. This system would make the thermal therapy more specific cause of the intrinsic feature of the cells to migrate towards the side of inflammation.

5.3 Release of therapeutic components from drug depots upon infections by synthetic circuits

The ability of the circuit to modulate inflammatory process could also be used to implement these cells to counteract bacterial infections upon implant transplantation. Here, the cells should be fixed locally along with the implant (e.g. implant cavities). This can be realized e.g. by encapsulation of the cells in alginate microparticles (Mazzitelli et al., 2011). Within these particles the cells are shielded from the host immune system while signals from the outside as well secretory proteins pass the particle thereby connecting the cells to and with the host system. IFN sensor cells such as Mx2-tTA cells could be used either to enhance the process of wound-healing or act as a sentinel to detect and counteract possible rising infections.

Currently, implant infections is one of the main reasons for implant failure. Classical therapies often fails since the establishment of biofilm makes bacteria insusceptible to antibiotic treatment (Zimmerli, 2014). So far strategies have been focused on the implant material itself to prevent the accumulation of microorganisms. The incorporation of anti-microbial compound (e.g. antibiotics) has several disadvantages. The amount which can be incorporated is limited and release is only mediated by diffusion. Consequently the drug depot will be depleted within short time. It could have been demonstrated before that bacterial infection itself on implant material lead to local IFN product (Rais et al., 2016). Here, IFN sensors such as the Mx2-tTA cells could detect the onset of excessive microbial growth and prevent the population on the implant. The incorporation of anti-microbial trigger (e.g. lysozyme) would break down bacterial cell walls and kill subsequently the bacteria (Figure 27).

This would be a direct approach towards anti-microbial activity. Another strategy focusing on a more indirect application would be the cotransplanation of loaded degradable release systems. Incorporation of drugs into lysozyme degradable chitosan nanocarrier could serve as drug depot system (Poth et al., 2015). The release of the compound would be realized only on demand via IFN signaling. The IFN mediated secretion of an enzyme (e.g. lysozyme) would be compatible with the formulation of the degradable nanocarriers similar to a lock-and-key model. This would subsequently lead to the release of the compound on demand interfering with onset of infection (Figure 27)

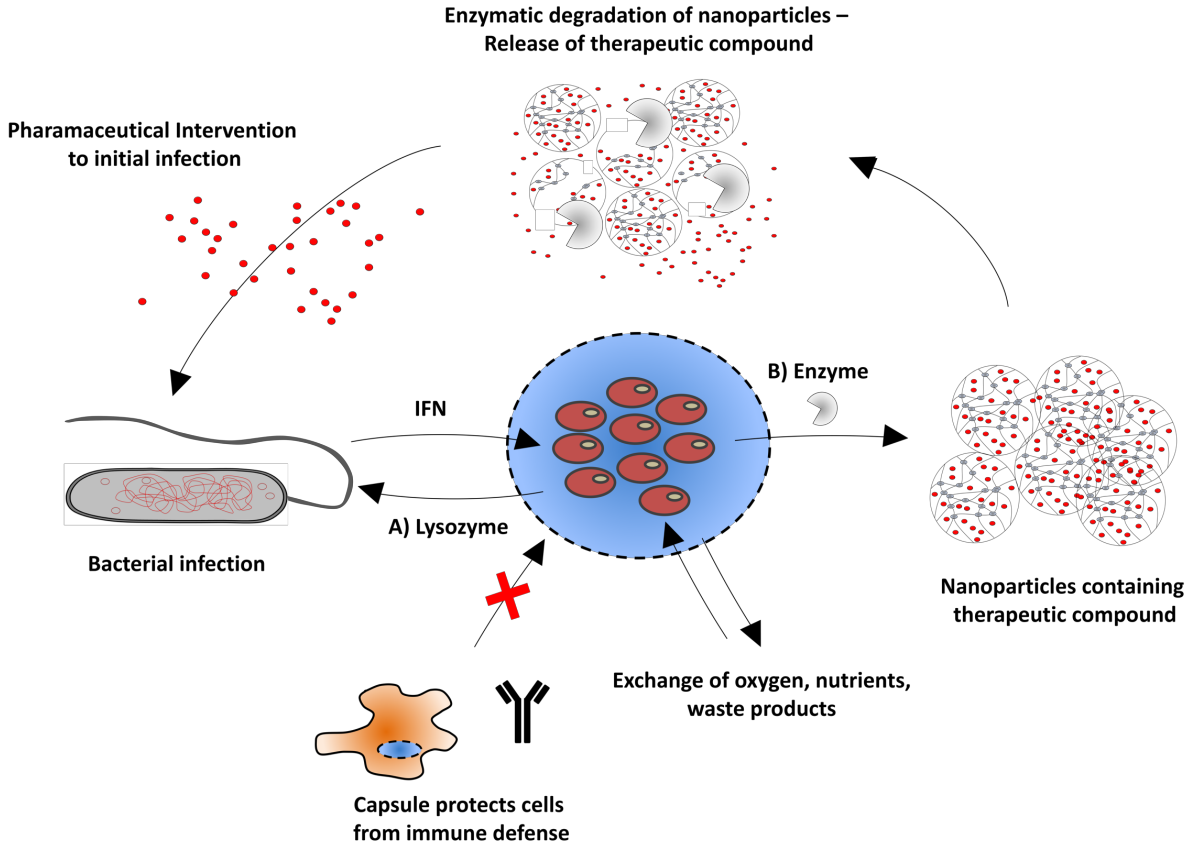

Figure 27: Schematic representation of potential therapeutic applications of IFN sensors.

The Mx2-tTA cells are encapsulated in alginate based microcapsules which ensures the exchange with nutrients, oxygen and waste products. Furthermore, these cells are shielded from the immune defense system. Upon a bacterial infection these cells could detect the infection by secreted IFN induced by tissue cells upon sensing the microbial patterns. A direct approach against the invading microorganism is the induced secretion of antibacterial proteins such as lysozyme by the encapsulated cells which breaks down the bacterial cell wall (A). An indirect approach would be the secretion of enzymatic triggers.. Such enzymes could specifically degrade nanoparticles that are loaded with therapeutic compounds, thereby releasing the therapeutic compound (e.g antibiotics) which would interfere with bacterial growth (B).

5.4 Alternative transactivator systems

The transactivator utilized to induce transgene expression in this study is one of various types of existing synthetic transcriptional activators. Once induced it can be repressed in the presence of doxycycline. In order to design circuits with a more precise resolution it might be of interest to use other kinds of transactivators. There are types of transcriptional activators which are regulated in the presence of e.g. light of a specific wavelength. These systems have been shown to work tightly with regard to temporal and spatial resolution (Polstein and Gersbach, 2012). Furthermore, it has been shown that engineered light regulatable cells are

effective in xenograft mouse models (Folcher et al., 2014). These cells could function as a synthetic defense mechanism upon transplantation of e.g. implants.

This study highlighted the challenge of stable expression of synthetic circuits in cells. With regard to the epigenetic silencing of the tTA promoter in long-term culture of murine embryonic stem cells different synthetic promoters could be employed to circumvent this transcriptional repression. Here LacI or Gal4 promoters could serve as possible alternatives in combination with their cognate DNA binding domain fused to an activator domain (Brown et al., 1987; Braselmann et al., 1997). However if these systems also underlie epigenetic silencing has to be tested. Alternatively, flanking the promoters with 'buffer' sequences that prevent inactivation might be an alternative.

5.5 Future perspectives

The treatment of human disorders irrespective of bacterial or genetic origin with drugs in the first place has been expanded to gene therapy strategies with progression of life sciences. Genetic networks established with principles of synthetic biology will offer another therapeutic approach. In the future patient´s derived cells might be engineered in order to restore organ function or to restore disturbed metabolic pathways. Moreover, these cells could be used to monitor physiological processes and inform the patient or even the physician when these shift to pathophysiological states. Smart phone based applications are already available and could be used to control the engineered cells activity (Shao et al., 2017). These kinds of applications could pave the way for a new kind of personalized translational medicine.

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8.Appendix

8.1 Abbrevations

Abbrevation	Explanation
BAC	Bacterial artificial chromosome
Bp	Basepair
CAR	Chimeric antigen receptors
cDNA	Complementary DNA
cGAS	Cytosolic GAMP synthase
CMV	Cytomegalovirus
CpG	CpG Dinucleotide
CRISPR	Clustered, regulatory, interspaced short palindromic repreats
crRNA	CRISPR RNA
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage-associated molecular pattern
DANN	Desoxyribonucleic acid
DIMx2-tTA	Differentiated, immortalized embryonic stem cell; tTA under Mx2 promoter control
DMEM	Dulbecco´s Modified Eagle´s Medium
DMSO	Dimethylsulfoxid
dNTP	Deoxynucleotide triphosphate
Dox	Doxycycline
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
Elf2α	Eukaryotic initiation factor 2α
ELISA	Enzyme linked immunesorbent assay
ER	Endoplasmatic reticulum
ESMx2-tTA	Embryonic stem cell; tTA under Mx2 promoter control
FACS	Fluorescence-β-actinactivated cell sorting
FCS	Fetal Calf Serum

G-418	Geneticin
gRNA	Guide RNA
HDR	Homology directed repair
HR	Homologous recombination
HRR	Homologous recombination repair
HSV	Herpes simplex virus
IFN	Interferon
IFN-β	Interferon beta
IFNAR	Interferon α/β receptor
IKK	IκB kinase
IL	Interleukin
iPSC	Induced pluripotent cells
IPTG	Isopropyl β-D-thiogalactopyranoside
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISG	Interferon stimualted gene
ISGF3	IFN stimulated gene factor 3
ISRE	IFN-stimulated response element
IVIS	*In Vivo* imaging system
JAK	Janus kinase
kb	Kiobase
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
Luc	Luciferase
MAR	Matrix attachment regions
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation associated gene 5
MEF	Mouse embryonic fibroblasts
mES	Mouse embryonic stem cell
Mx	Mx dynamin like GTPase
NFκB	Nuclear factor κB

NHEJ	Non homologous end-joining
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
OD	Optical density
PAM	Protospacer adjacent motif
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PKR	IFN-inducible dsRNA-activated protein kinase
polyI:C	Polyinosinic:polycytidylic acid
PRD	Positive regulatory factor
PRR	Pattern recognition receptor
qRT-PCR	Quantitative Real Time PCR
RFP	Red fluorescent protein
RIG-I	Retinoid acid-inducible gene I
RLR	RIG-I-like receptors
RLU	Relativ light untis
RNA	Ribonuleic acid
rpm	Rounds per minute
RT-PCR	Real Time PCR
rtTA	Reverse tetracycline transactivator
sgRNA	Single-guide RNA
SOCS1	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
SV	Simina-Virus
TAM	Tumor associated macrophages
TBK1	TANK-binding kinase 1
TEM	Tumor microenvirnoment

TEP	Trypsion EDTA
TetO	Tetracycline operator
tetR	Tetracycline repressor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
tracrRNA	Trans-activting RNA
TRE	Tetracycline-response promoter element
tTA	Tetracycline transactivator
Tyk2	Tyrosine kinase 2
UCOE	Ubiquitous chromatin opening element
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VP16	Herpes simplex virus transcriptional regulatory protein
X	Times
ZALEN	Transcription activator-like effector nuclease
ZFN	Zinc finger nuclease

8.2 List of Figures

8.3 List of Tables

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